Genetic analysis of the host interactions of Photorhabdus

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Genetic Analysis of the Host Interactions of *Photorhabdus*

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List of Abbreviations

Amp	Ampicillin
AMP	Adenosine monophosphate
APS	Ammonium persulphate
Bp	Base pairs
°C	Degrees Celcius
cAMP	Cyclic AMP
CAS	
CFU(s)	Colony forming unit(s)
CIAP	Calf intestinal alkaline phosphate
CRP	cAMP receptor protein
cm	Centimetre
dH_2O	Distilled water
dsH_2O	Distilled sterile water
DNA	Deoxyribosenucleic acid
dNTP	Deoxynucleotide 5'-triphosphate
EDTA	disodium ethylenediaminetetra acetate.$2H_2O$
EMB	Eosin mythyl blue
Fig.	Figure
g	Gram
h	Hour(s)
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethansulphonic acid
IJ(s)	Infective juvenile(s)

Kan	Kanamycin
kb	kilobase
l	Litre
LB	Luria-Bertani
LD_{50}	Lethal dose
LT_{50}	Lethal time
LIA	Lipase indicator agar
LPS	Lipopolysaccharide
M	Molar
mA	Milliamps
Mb	Megabase
MES	2-(N-Morpholino)ethanesulphonic acid
Min(s)	Minute(s)
mg	Milligrams
ml	Millilitre
mM	Millimolar
MW	Molecular weight
µg	Microgram
µl	Microlitre
µM	Micromolar
ng	Nanogram
NBTA	Nutrient bromothymol blue agar
OD	Optical density
Oligo	Oligonucleotide
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

pH	$-\log_{10}[H^+]$
PVC	Polyvinyl chloride
Rif	Rifampicin
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride sodium citrate
Spp	Species
Subsp	Subspecies
TAE	Tris-acetate EDTA
TEMED	N, N, N', N',- tetramethyl ethylene diamine
TEM	Transmission electron microscopy
Tris	Tris(hydroxymethyl)aminomethane
U	Unit
V	Volts
v/v	Volume/volume
w/v	Weight/volume

Abstract

Photorhabdus spp. (Enterobacteriaceae) are bacterial symbionts of the entomopathogenic nematode *Heterorhabditis*, colonising the gut of the infective juvenile stage. The nematodes infect a variety of soil dwelling insects releasing the bacteria into the insect haemolymph where the bacteria multiply killing the insect and converting the insect body mass into a substrate on which the nematodes feed and subsequently develop and reproduce.

The importance of bacterial motility in symbiotic and pathogenic host interactions has been shown in numerous models. In this study we examine the role of motility in the symbiosis and pathogenicity of *Photorhabdus luminescens*. Using transposon mutagenesis we isolated mutants with varying degrees of motility, which were screened for their ability to support nematode growth and for insect virulence. A non-motile aflagellate mutant BMM316 was identified and shown to have a transposon insert in the flagella structural gene *flgG*. Interestingly this mutation was shown to have no effect on the pathogenicity of *P. luminescens* towards *Galleria mellonella* larvae. However BMM316 was shown to be deficient in colonisation of the nematode partner, therefore flagella meditated motility is important in the retention of *Photorhabdus* by *Heterorhabditis*.

A second mutant, BMM305, with relatively minor motility defects was also isolated and characterised. The insert was shown to disrupt the *pbgE1* gene, which is homologous to *pmrK* in *Salmonella*. BMM305 was severely attenuated in both pathogenicity and symbiosis indicating that *pbgE1* is essential for the interactions of *P. luminescens* with both hosts. BMM305 was further characterised and shown to lack an O antigen and exhibited sensitivity to mildly acidic pH and polymyxin B. The results obtained during this study suggest that the O antigen is essential for colonisation of both hosts.

CHAPTER 1

1.0 Introduction.

1.1 General Introduction.

1.1.1 *Photorhabdus*

Photorhabdus is a genus of motile Gram-negative bacteria belonging to the family *Enterobacteriaceae* (Boemare *et al.*, 1993). There are three species of *Photorhabdus*, *P. luminescens*, *P. temperata* and *P. asymbiotica* (Fischer-Le Saux *et al.*, 1999). With the exception of the clinical isolates designated *P. asymbiotica,* isolated in America and Australia (Farmer *et al.*, 1989; Peel *et al.*, 1999), all *Photorhabdus* species isolated so far have been identified in symbiotic association with the entomopathogenic nematodes belonging to the family *Heterorhabditidae* (Forst and Clarke, 2002). This symbiotic association forms the basis for a pathogenic complex able to infect and kill a wide range of soil dwelling insects (Forst and Clarke, 2002).

1.1.2 Life Cycle of *Photorhabdus*

Although *Photorhabdus* can be cultivated on standard media in the laboratory, it appears that in the wild its association with *Heterorhabditis* is obligatory, as *Photorhabdus* bacteria have not yet been isolated without their respective nematode hosts, with the exception of *P. asymbiotica.* Furthermore *Photorhabdus* survive poorly in soil with approximately a 0.004% survival rate after 7 days (Bleakley and Chen, 1999). Therefore *Photorhabdus* requires the nematode for protection from the soil and infection into the insect host. The nematodes need an environment in which they can develop, reproduce and complete their life cycle, and the bacterial partner provides this environment. Furthermore, axenic *Heterorhabditis* nematodes are unable to complete their life cycle *in vivo* and *in vitro* (Han and Ehlers, 2000); therefore *Photorhabdus* is essential for *Heterorhabditis* development and *Heterorhabditis* are essential for the protection and survival of *Photorhabdus*.

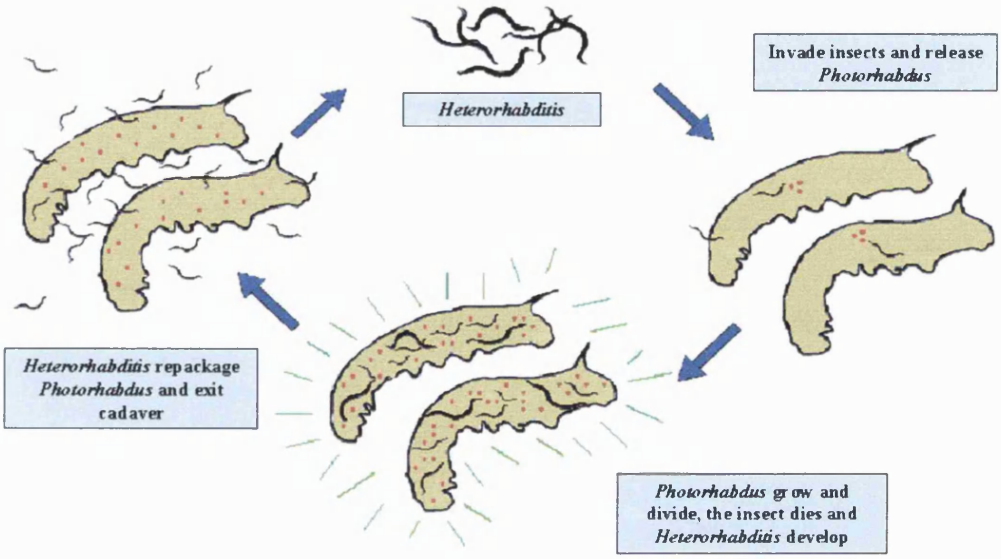


Figure 1.0. The life cycle of the entomopathogenic nematode *Heterorhabditis* and its bacterial symbiont *Photorhabdus*.

In the soil *Photorhabdus* are found in the midgut of the infective juvenile (IJ) stage of *Heterorhabditid* nematodes (Ciche and Ensign, 2003). The IJ is a specialised stage of nematode development that is adapted to survive for long periods in the soil where it seeks out insect hosts and invades through natural openings such as the mouth, anus or spiracles. In addition *Heterorhabditis* nematodes posses an anterior tooth that allows the IJ to penetrate directly through the cuticle (Akhurst and Dunphy, 1993). Once inside the insect *Heterorhabditis* actively regurgitates *Photorhabdus* cells, and *in vitro* this occurs approximately 30mins after exposure to insect haemolymph (Ciche and Ensign, 2003). *Photorhabdus* spp. are highly pathogenic with a low LD_{50}, and it appears that the bacteria can evade the insect immune system and grow and divide to high numbers that ultimately result in insect death within approximately 48h (Daborn *et al.*, 2001). The LD_{50} and LT_{50} vary between bacterial strains, the insect host species and its physiological state (Akhurst and Dunphy, 1993).

Inside the insect *Photorhabdus* releases numerous extracellular products that damage or break down the insect tissues and organs. This breakdown eventually bioconverts the insect body mass into a substrate on which the nematodes can develop. Bacterial products responsible for this are likely to include LPS endotoxin (Clarke and Dowds, 1995), the protein product of *mcf* (Daborn *et al.*, 2002), insecticidal toxins (Bowen *et al.,* 1998) and enzymes that include haemolysin, lipases and proteases (Brillard *et al.*, 2002; Wang and Dowds, 1993; Boemare and Akhurst, 1988; Schmidt *et al.,* 1988).

Competition inside the insect needs to be kept to a minimum; this is initially achieved by the insect's own immune response eliminating non-symbiotic bacteria on the nematode body surface. *Photorhabdus* can escape the insect immune system and during later stages of insect infection the bacteria produce potent antibiotics that inhibit the natural gut flora and probably help to keep the insect from putrefying (Hu and Webster, 2000). This keeps the insect cadaver in favourable conditions for the persistence of the specific bacteria-nematode complex. Furthermore *Photorhabdus* produce an ant deterrent factor that discourages the scavenging of the insect cadaver; thus protecting themselves and the developing nematode partners (Zhou *et al.*, 2002).

There are two food signals that indicate to the IJ to recover and start development within the insect host. The first is present in the haemolymph (Han and Ehlers, 2000), and the second is secreted by the bacterial partner (Aumann and Ehlers, 2001). Upon the appropriate food signal *Heterorhabditis* IJs recover into self-fertile hermaphrodites, which lay eggs that develop into males and females (Forst and Clarke, 2002). The developing nematodes feed on the broken down components of the insect body and the bacterial cells. Several rounds of reproduction occur and upon a dwindling food signal, or a signal inducing IJ formation from the bacterial partner, the nematodes exit their life cycle and develop into the alternative stage three larvae – the IJ (Aumann and Ehlers, 2001). As they develop into IJs they selectively repackage and retain their bacterial symbiont.

Photorhabdus also has the unique ability amongst terrestrial bacteria to bioluminesce. *Photorhabdus* emit a blue-green light at stationary phase both *in vivo* and *in vitro*, which is due to the possession of the *lux* operon consisting of *luxCDABE* (Frackman *et al.*, 1990; Daborn *et al.*, 2001). The luciferase enzyme (encoded by *luxAB*) uses molecular oxygen to convert two substrates, an aldehyde and a reduced flavin, and light is a by-product of this reaction. The aldehyde is provided by another enzyme, fatty acid reductase, (encoded by *luxCD*) (Frackman *et al.*, 1990). Although the mechanism of light production has been elucidated its role remains unclear. It is known that luciferase has a very high affinity for O_2, and it has recently been suggested luciferase has a scavenging role for O_2, thus protecting the bacteria and the nematode against damaging reactive oxygen species (ffrench-Constant *et al.*, 2003).

1.1.3 *Xenorhabdus*, Differences and Similarities to *Photorhabdus*

A close relative of *Photorhabdus* is *Xenorhabdus*; the bacteria share many similarities and have only in the last decade been identified as two separate genera (Boemare *et al.*, 1993). The host interactions and life cycle of *Photorhabdus* and *Xenorhabdus* are very similar; both genera are bacterial symbionts of a nematode partner and both are highly pathogenic for a range of insect hosts. Despite the similarities in the two systems several differences can be observed, both in the development of the nematode and the phenotypic characteristics of the bacterial partner. Analysis of these disparities has lead to the hypothesis that these symbiotic associations have arisen separately and represent convergent evolution (Forst and Nealson, 1996).

In vivo the development of *Heterorhabditis* nematodes is absolutely dependent on the presence of *Photorhabdus* as axenic *Heterorhabditis* nematodes are not capable of killing the insect host *Galleria mellonella*. Therefore, insect death is an important prerequisite for *Heterorhabditis* development, and only partial nematode development is seen in the absence of *Photorhabdus* (Han and Ehlers, 2000). However the requirement of *Steinernema* for *Xenorhabdus* is less restrictive and axenic *Steinernema* are capable of killing *Galleria mellonella* and completing their life cycle to a limited degree (Han and Ehlers, 2000). Further differences involving the nematode include the location of bacteria inside the IJ.

Photorhabdus bacteria are located in the anterior midgut region of *Heterorhabditis* IJ nematodes, whereas *Xenorhabdus* are found in a developmentally specialised sac in the gut of *Steinernematid* IJ nematodes (Forst and Clarke, 2002). Finally, *Heterorhabditis* develop from IJs to self-fertile hermaphrodites, whereas *Xenorhabdus* recover from IJ and develop into either male or females before undergoing further reproduction (Forst and Clarke, 2002). Therefore, at least two *Xenorhabdus* IJs are required for successful completion of their life cycle.

Furthermore, *Xenorhabdus* produces an array of enzymatic activities that are detrimental to insect larvae and has phenotypic traits that are superficially similar to *Photorhabdus* (Boemare and Akhurst, 1988). However closer analysis reveals disparities as summarised by Forst and Clarke (2002) and further suggests the traits essential for an entomopathogenic lifestyle were divergently acquired. Lastly it has been suggested based on morphological studies that the nematode partners of *Photorhabdus* and *Xenorhabdus*, *Heterorhabditis* and *Steinenema* respectively, have divergent ancestral origins and may also represent convergent evolution (Poinar, 1993). In summary, despite the similarities between the *Photorhabdus/Heterorhabditis* and *Xenorhabdus/Steinernema* symbiosis, several phenotypic and genetic differences are apparent and the likely separate evolutionary origins need to be considered when comparing the two systems.

1.1.4 Phenotypic Variation and Regulation

Upon prolonged *in vitro* culture both *Photorhabdus* and *Xenorhabdus* undergo phenotypic variation to a secondary variant. The primary variant is pathogenic and supports the growth and development of the nematode partner whereas the secondary variant is pathogenic but non-symbiotic (Akhurst, 1980). The primary variant is also associated with many of the characteristics of *Photorhabdus* and *Xenorhabdus* including typical cell and colony morphology, dye uptake, motility, production of exoenzymes, crystalline inclusion proteins and antibiotics. The secondary variant lacks or is greatly reduced in many of these characteristics (Akhurst, 1980; Boemare and Akhurst, 1988; Bleakley and Nealson, 1988; Givaudan *et al.*, 1995). These differences have lead to the hypothesis that the

characteristics present in the primary variant are associated with the ability to support symbiosis (ffrench-Constant *et al.*, 2003).

A great deal of work has been carried out into the cause, control and effect of *Photorhabdus* phenotypic variation; however a definitive answer as to how and why phenotypic variation occurs has not yet been elucidated. It has been suggested that the secondary variant is better adapted for life in the soil as it has a shorter lag phase and is more efficient at nutrient uptake than primary variants (Smigielski *et al.*, 1994). Secondary variants of *Photorhabdus luminescens* also have a shorter generation time possibly due to the fact they do not produce secondary metabolites and can divert more energy to growth and cell division (Bleakley and Nealson, 1988). Therefore, if a secondary variant was in the nutrient poor environment of the soil and was transported to a nutrient rich environment, a short lag phase and short generation time would give a distinct advantage over other micro-organisms. This hypothesis therefore suggests the secondary phase may be better adapted for life in the soil without the nematode.

The secondary variants of both *Photorhabdus* and *Xenorhabdus* have not yet been isolated from free-living nematodes or the soil; therefore the appearance of these variants remains a laboratory-induced phenomenon. However, Krasomil-Osterfeld (1995) demonstrated that, for *Photorhabdus* at least, one environmental factor, low osmolarity, could induce the appearance of secondary variants suggesting a possible *in vivo* mechanism for phenotypic variation. Furthermore, reversion from secondary to primary variant was possible if exposure to low osmolarity was not prolonged. Intriguingly *Photorhabdus* is also able to produce small colony variants. These variants show both similarities and differences to primary variants and can switch to either primary or secondary at a high frequency (Hurlbert *et al.*, 1989; Hu and Webster, 1998; Gerritsen and Smits, 1992). The discovery of these variants suggests the mechanisms controlling phenotypic variation are numerous and complex. In addition, several recent studies have shown that numerous loci affect phenotypic variation in different ways. The over-expression of the regulatory gene *pln* in primary variants of *Photorhabdus* causes the cells to appear secondary-like, although a knockout in secondary variants did not cause them to express primary phenotypes (Roche,

2002; O'Neill *et al.*, 2002). Mutations in *Photorhabdus* crystalline inclusion body proteins (CIPs) produced strains that appeared to be between primary and secondary variants for many characteristics (Bintrim and Ensign, 1998). A knockout of the *Photorhabdus* regulatory gene *hexA* in secondary variants induced primary like characteristics, with the exception of motility (Joyce and Clarke, 2003). Finally, 127 primary *Photorhabdus* transposon mutants were obtained that exhibited secondary-like pigmentation; many of these mutants were not secondary-like for any other phenotypes. However one mutant was identified as being in a LysR type regulator and appeared to be very secondary-like (H. McWeeney and J. Williams, personal communications).

Interestingly, a two-component pathway has recently been identified in *Photorhabdus* to have a role in adaptation to stationary phase and phenotypic variation. The pathway AstRS has been shown to regulate motility, antibiotic production, energy metabolism, iron acquisition and stress responses (Derzelle *et al.*, 2004b). Although the two-component pathway shares similarity with the BvgAS system of *Bordetella* the signals that activate the BvgAS system do not activate AstRS. The data suggested links between stationary phase and phenotypic variation, however the precise role of AstRS in the life cycle of *Photorhabdus* remains unclear as an *astR* mutant remained virulent, and the interaction with the nematode host was not investigated. This data further suggests phenotypic variation is a complex and branched signalling cascade.

1.1.5 Phenotypic Variation in Pathogenicity and Symbiosis

Photorhabdus provides a tool for the study of symbiosis and pathogenicity in one organism. Further, the phenomenon of phenotypic variation associated with the loss of symbiosis and several major characteristics provides the opportunity for investigation into the role these characteristics play in bacterial-host interactions. Several studies have already been undertaken to look at the role various genes and characteristics have in both the symbiosis and pathogenicity of *Photorhabdus* and *Xenorhabdus*.

The LPS toxins and exoenzymes that *Photorhabdus* and *Xenorhabdus* produce and release during insect infection have naturally been suggested as virulence

factors. For *Photorhabdus* however, LPS alone has proved to be non-lethal upon injection (Clarke and Dowds, 1995). Furthermore a *phlA* (haemolysin) mutant was shown to be unaffected in virulence (Brillard *et al.*, 2002), specific toxins and the *ptrA* protease are also produced *in vivo* after insect death (Daborn *et al.*, 2001) and strains of *Photorhabdus* secondary variants, which are as pathogenic as primary variants, are deficient in general lipase and protease activity (Bleakley and Nealson, 1988). These results suggest that the majority of the breakdown of the insect body occurs after death, and that the likely cause of death is a combination of septicaemia and specific toxin action. The toxin Tcd is produced at approximately 18h, which is significantly before insect death and likely to be a contributory factor (Daborn *et al.*, 2001).

A model has been proposed whereby factors produced in exponential phase *in vivo* (before insect death) are important in pathogenicity, and factors produced in stationary phase *in vivo* (after insect death) are important in symbiosis (ffrench-Constant *et al.*, 2003). This model corroborates the data provided and further suggests that primary specific factors are in fact symbiosis factors. Moreover, primary variants possess these factors and are symbiotic and secondary variants generally do not possess them and are non-symbiotic. Therefore although both primary and secondary variants can grow inside the insect larva and cause death it appears that only the primary variant can then fully bioconvert the larva into appropriate substrates on which the nematodes can develop.

In support of this model, Hu and Webster (2000) have shown that the appearance of hermaphrodites does not occur until at least 120h post infection (72h after insect death). This indicates that nematode development does not start to occur until at least 3 days after insect death, when the cadaver is likely to be being broken down. The supporting of symbiosis by providing a suitable substrate is likely to occur in synchrony with a factor that the nematode can recognise in the primary, but that is absent in secondary variants. This second bacterial related factor has been suggested as some secondary strains will support nematode growth to a limited extent *in vitro* (when the nematode is being supplied with food); however development is restricted and the secondary variants are rarely repackaged (Han and Ehlers, 2000).

1.1.6 The Regulation of Symbiosis in *Photorhabdus* and *Xenorhabdus*

Several studies have been undertaken to try and elucidate the mechanisms of symbiosis in *Photorhabdus* and *Xenorhabdus* including random transposon mutagenesis and signature tagged mutagenesis. These studies have revealed several genes with significant roles in symbiosis including *ngrA*, *rpoS* and *hexA* (Ciche *et al.*, 2001; Heungens *et al.*, 2002; Joyce and Clarke, 2003).

The symbiotic association of *Photorhabdus* and *Xenorhabdus* with their nematode partners can be divided into two events. Firstly, the ability of the bacteria to provide suitable substrates for the nematode to develop on, and secondly, the ability of the bacteria to colonise the IJs that emerge after successful growth and development of the nematode partner. *In vivo* the ability to provide suitable substrates for the nematode means being able to kill and convert the body mass of an insect host; therefore pathogenicity is a pre-requisite for symbiosis. The colonisation of the nematode partner is itself likely to be a two-step process as recent data for the *Xenorhabdus* and *Steinernema* symbiotic association suggests (Martens *et al.*, 2002). The model proposed is that one or two *Xenorhabdus* cells are retained by the nematode and that these cells grow inside the lumen of the IJ intestine to form the final population. Recent work suggests that this is also the case for *Photorhabdus* (R. Watson, personal communication). Thus symbiosis is a highly complex event with many stages and signals that need to be regulated accordingly.

The sigma factor σ^S is encoded by the gene *rpoS* and is a key global regulator involved in mediating bacterial responses to stresses such as osmolarity, acid shock, heat shock and the onset of stationary phase (Hengge-Aronis, 2000). Therefore σ^S is involved in bacterial-host interactions largely through the ability of bacteria to survive host mediated stresses (Chen *et al.*, 1997; Yildiz and Schoolnik, 1998). However in *Salmonella* σ^S can affect virulence directly through the regulation of the plasmid encoded virulence genes *spv*, which are required for the growth of *Salmonella in vivo* (Hengge-Aronis, 2000; Loewen *et al.*, 1998; Nickerson and Curtiss, 1997). Interestingly an *rpoS* knockout in *Xenorhabdus* retained its virulence and was able to support the growth and development of its nematode partner. However it was not able to re-colonise the

IJ intestinal vesicle suggesting a possible environmental stress present in the nematode that was not overcome (Vivas and Goodrich-Blair, 2001). Therefore in *Xenorhabdus* σ^S is likely to represent a global regulator involved in symbiotic associations with the nematode host. In *E. coli* however, σ^S controls the transcription of approximately 50 genes (Loewen *et al.*, 1998) and work in *Salmonella* has identified genes regulated by σ^S with no homology to the genes so far identified in *E. coli* (Ibanez *et al.*, 2000). Therefore σ^S is likely to regulate numerous genes in *Xenorhabdus* and the precise role it has in symbiosis has yet to be elucidated. Interestingly, although the phenotypic characteristics of *Xenorhabdus* were not affected by the mutation, motility was up regulated. The appropriate regulation of motility is important in bacterial host interactions (Otterman and Miller, 1997), and the inappropriate expression of flagella has a detrimental effect on the virulence of *Bordetalla* towards its host (Akerley *et al.*, 1995). The improper up regulation of motility may therefore also have disadvantageous effects on the symbiotic host interactions of *Xenorhabdus* with *Steinernema.*

Another putative transcriptional regulator has recently been identified as being involved in the regulation of symbiosis in *Photorhabdus* (Joyce and Clarke, 2003). The gene *hexA* had previously been identified as being important in the negative regulation of extracellular proteins in the plant pathogen *Erwinia carotovora* (Harris *et al.*, 1998). A *hexA* mutation in the secondary variant of *Photorhabdus temperata* was shown to restore the ability to support growth and development of the nematode partner and restore many of the phenotypic characteristics normally associated with the primary variant. This suggests *Photorhabdus hexA* is repressing the production of many extracellular products in manner similar to *hexA* in *E. carotovora.* However *hexA* in *E. carotovora* also regulates motility whereas *hexA* in *Photorhabdus* was shown not to have an effect on motility (Joyce and Clarke, 2003; Harris *et al.*, 1998).

Interestingly in *E. carotovora hexA* negatively regulates the production of *rpoS* (Mukherjee *et al.*, 2000), and the expression of *hexA* in *Photorhabdus* is associated with a decrease in the levels of σ^S. However in the absence of *hexA* expression *rpoS* levels do not return to parental levels indicating other factors

may work to repress *rpoS* in the secondary *Photorhabdus* variant (Joyce and Clarke, 2003). Furthermore it is unlikely HexA regulates the production of primary characteristics through σ^S, as a *Xenorhabdus rpoS* mutant was not affected in its primary characteristics (Vivas and Goodrich-Blair, 2001).

1.1.7 Symbiotic Signals in *Photorhabdus*

A specific biochemical enzyme has been isolated as being essential for *Photorhabdus* to support the growth and development of *Heterorhabditis*. The gene *ngrA* encodes a protein with homology to a 4'-phosphopantetheinyl (Ppant) transferase and mutants in *ngrA* consistently failed to support the development of nematodes (Ciche *et al.*, 2001). Ppant transferases are involved in the production of fatty acids, polyketides and non-ribosomal peptides. The transfer of a Ppant moiety activates the carrier proteins involved in the construction of these large metabolites (Mofid *et al.*, 2002). In *E. coli* a Ppant transferase (EntD) is required for the synthesis of the siderophore enterobacterin (Gehring *et al.*, 1997). Interestingly, the *ngrA* mutant was defective in siderophore and antibiotic production, although recent data suggests that it is not the lack of siderophore that is the cause of the symbiotic defect (Ciche *et al.*, 2003). Indeed the authors postulate the existence of an undiscovered secondary metabolite product of Ppant transferase that is essential for growth and development, and possibly functions as a symbiotic signal to the nematodes.

Ppant transferases can be divided into two groups; one specifically involved in fatty acid biosynthesis and one involved in the production of a broad range of substrates (Moftid, *et al.*, 2002). The product of *ngrA* is likely to be in this second class, as *Photorhabdus* has a gene (*acpS*) that is likely to be involved in fatty acid biosynthesis. However the broad range of substrates involved in the second class means *ngrA* may still act to produce fatty acids. The product of the gene *fadD* affects degradation of fatty acids and a *Sinorhizobium meliloti fadD* mutant was affected in symbiosis. Therefore a fatty acid derivative has been hypothesised to act as an intracellular signal controlling symbiosis (Soto *et al.*, 2002). Furthermore, a mutation in a *fadD* homologue in *Salmonella* affects invasion of host cells and the authors also suggest derivatives that may act as intracellular signals for host interactions (Lucas *et al.*, 2000). Therefore NgrA

may affect the production of a fatty acid and this fatty acid may be involved in intracellular signalling; perhaps for symbiosis.

1.1.8 Colonisation in *Xenorhabdus*

Photorhabdus and *Xenorhabdus* are found in the intestine of their respective nematode hosts and the locations indicate close physical associations between bacterial and nematode cells. Furthermore these close interactions are likely to be mediated by physical appendages on the bacterial cell surface such as flagella, pili, fimbrae, membrane proteins or LPS. Bacterial cell surface structures have been well documented as having roles in close host interactions. Flagella have been implicated in initialising attachment during biofilm formation of Gram-negative bacteria, which are associated with many bacterial infections (O'Toole *et al.*, 2000; Singh *et al.*, 2000). In addition type IV pili are essential for twitching and this has been shown to be important for microcolony formation during biofilm development (O'Toole and Kolter, 1998). Furthermore, correct LPS structure has been shown to be necessary for the symbiosis of *Sinorhizobium* with alfalfa (Campbell *et al.*, 2002). Therefore these physical appendages represent an interesting target for research into the interactions of *Photorhabdus* and *Xenorhabdus* with their respective nematode hosts.

Heungens *et al.*, (2002) recently isolated 4 transposon mutants in a 4kb locus that were all attenuated in their ability to colonise the specialised vesicle of the *Steinernema* IJ. These mutants were given the designation *nil* (nematode intestine localisation) and two of the mutants, *nilB* and *nilC*, were hypothesised to encode outer membrane proteins with unknown homology to any protein in the database. Further, *nilC* has regions of homology with flagella and pilin proteins suggesting a putative external structure and a role in physical bacterial-nematode contact. The authors suggest these structures may mediate specificity between *Xenorhabdus* and *Steinernema*.

Three transcriptional regulators as well as two enzymes were also identified as being important in colonisation of the *Xenorhabdus* IJ; although the precise role of these genes is still unknown (Heungens *et al.*, 2002). The identification of transcriptional regulators and biochemical processes important in symbiosis will

broaden our understanding of bacterial nematode interactions. Furthermore, the physical external structures of bacterial cells are likely to be important in this symbiosis and the study of these structures offers a deeper understanding into both symbiotic and pathogenic interactions of *Photorhabdus* and *Xenorhabdus*.

1.1.9 Application

Entomopathogenic nematodes are used as a biocontrol agent. They are ecologically friendly and their effectiveness against insect pests has led to their widespread use and mass production (Ehlers, 2001). Secondary variants of *Photorhabdus* and *Xenorhabdus* do not support the growth and reproduction of their nematode partners and effect nematode yields *in vitro*. Therefore many questions have arisen as to the nature of the symbiotic potential of primary and secondary variants. An understanding of the system governing specificity of association may provide insights into allowing different combinations of bacteria and nematode to complement nematode host finding behaviour and bacterial virulence. This could allow for increasing or narrowing host ranges and making the nematode-bacteria complex a more malleable tool. As *Photorhabdus* is able to kill a wide range of insect hosts research into the toxins that cause insect mortality is helping to produce pest resistant transgenic crops whilst also shedding light on the nature of *Photorhabdus* and *Xenorhabdus* pathogenicity (Liu *et al.*, 2003; Bowen *et al.*, 1998; Daborn *et al.*, 2002).

1.2 Bacterial Motility

Bacterial motility can be divided into 3 categories; swimming, swarming and gliding. Swimming and swarming are mediated by flagella. Swimming is the movement through liquid or semi-solid media, whilst swarming is the movement over solid surfaces. Gliding is movement without flagella and one form of gliding is called twitching. Twitching is surface associated and mediated by extension and retraction of type IV pili (Skerker and Berg, 2001). There is no definitive role for flagella or motility in the life cycle of bacteria and if a strain has the capacity to express flagella and pili genes it may only do so at certain times in specific environments. The precedence is that active motility, mediated by flagella and pili, is important in both virulent and symbiotic host interactions. The pathogens *Vibrio anguillarum* and *Pseudomonas aeruginosa* both require

active flagella for proper host invasion or infection, whilst the symbiont *Vibrio fischeri* requires active flagella for its symbiotic associations (Feldman *et al.*, 1998; O'Toole *et al.*, 1996; Graf *et al* 1994). However several virulent bacterial-host interactions require that motility is switched off or appropriately regulated for full pathogenesis. The pathogen *Bordetella* actively represses flagella transcription inside its host (Akerley *et al.*, 1995; Otterman and Miller, 1997). Furthermore some important pathogens are non-motile and do not express flagella. *Yersinia pestis*, the causative agent of the plague, is non-motile despite containing two sets of flagella genes – one homologous to the *E. coli* and *Salmonella* flagella systems and the other, which is not complete, is only distantly related to other characterised systems (Deng *et al.*, 2002).

1.2.1 Swimming

Bacterial swimming is a tactic response of numerous species in semi-solid or liquid media and it is mediated by flagella. The flagellum consists of a rotating helical filament protruding from the cell that is driven by a motor at its base (Macnab, 1996). The flagellum also contains a switch mechanism and thus the filament can rotate clockwise (CW) or counter clockwise (CCW). CCW rotation results in flagella forming a tight bundle and propelling the cell in a forward swimming motion. CW rotation causes the bundle to be dispersed and the cell tumbles randomly. The cell can randomly 'walk' through its environment using alternate swimming and tumbling modes. Closely associated with this motor function of the flagella is the chemotaxis system. Chemotaxis is the sensor system that, in conjunction with the motor systems, allows tactic responses to be made to stimuli. In response to a positive stimulus, or the reduction in negative stimuli, the bacterial cell can increase the time it swims for resulting in a net movement towards the positive stimuli or away from the negative (for a review see Blair, 1995).

The flagella structure (Fig 1.1) includes a basal body, which traverses the bacterial membranes and includes rings of protein associated with layers of the Gram-negative cell wall. The MS ring is associated with the inner membrane, the P ring with the periplasm and the L ring with the outer membrane. Essential to flagella function are the motor, which is associated with the peptidoglycan and

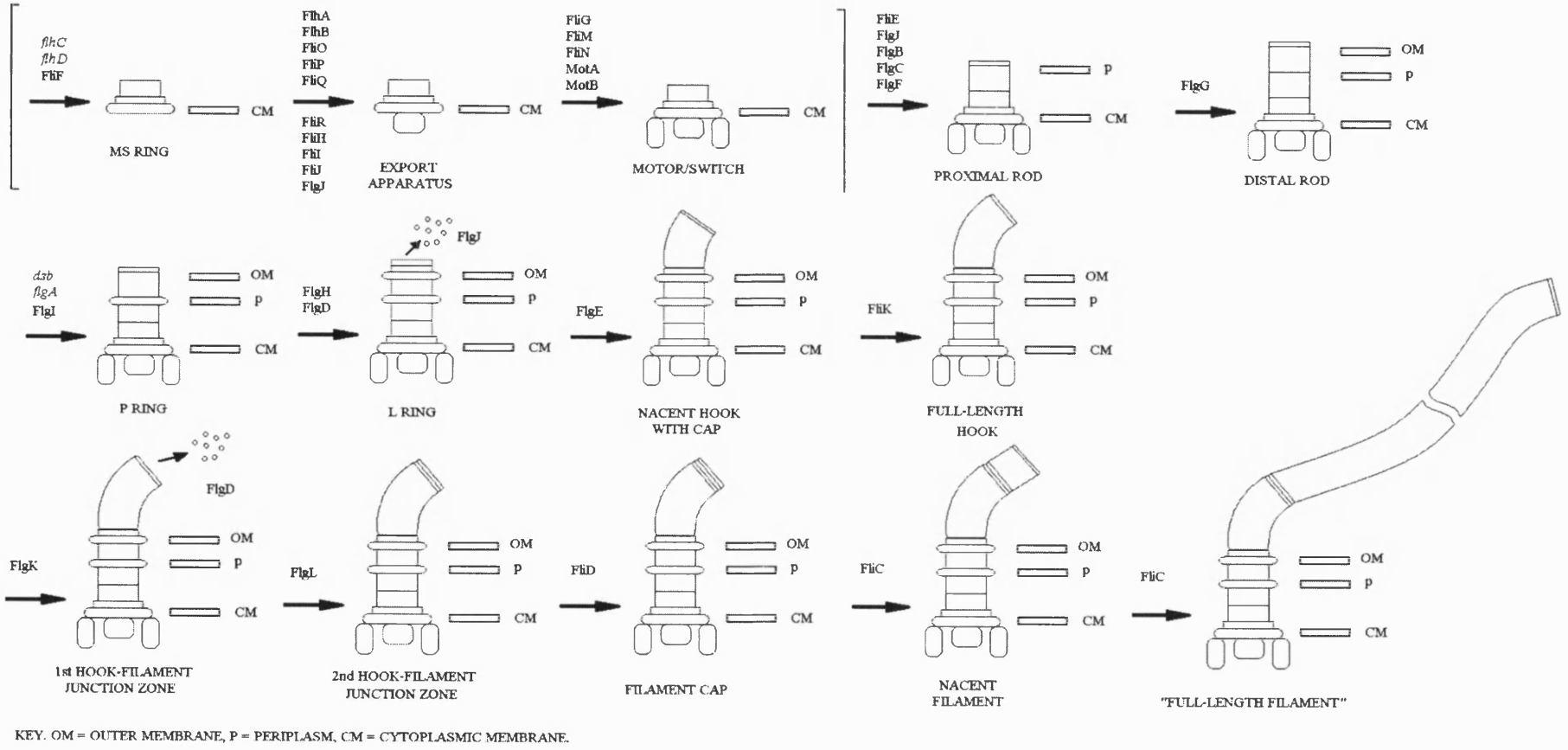


Figure 1.1. Assembly of flagella in *Salmonella*. Brackets indicate substructures assembled prior to utilisation of flagella export pathway. Genes (italics) or proteins necessary at each stage are indicated. Modified from Macnab (2003).

Operon /gene and class in *E. coli*		**Homologue in *Photorhabdus*?**
flhDC	Class I	*flhDC*
flgA	Class II	*flgA*
flgBCDEFGHIJ	Class II	*flgBCDEFGHIJ*
flhBAE	Class II	*flhBA*
fliA	Class II	*fliAZ*
fliE	Class II	*fliE*
fliFGHIJK	Class II	*fliFGHIJK*
fliLMNOPQ	Class II	*fliLMNOPQ*
fliR	Class II	*fliR*
flgMN	Class III	*flgMN*
flgKL	Class III	*flgKL*
motAB,cheAW	Class III	*motAB,cheAWD,plu1854,*
tar,tap,cheRBYZ	Class III	*cheRBYZ*
fliC	Class III	*fliC*
fliDST	Class III	*fliDST*
Total = 48 genes		**Total = 48 genes**

Table 1.0. List of operons and genes involved in the production of flagella in *E. coli*, their class and their homologues in *Photorhabdus*.

inner membrane area of the basal body, and the switch that is also associated with the inner membrane. Structurally important are the hook, which is present at the distal end of the basal body and the flagella filament, which when rotated provides thrust (Macnab, 2003).

Flagellar mediated motility and chemotaxis are complex, highly regulated phenomena that are also energetically expensive involving approximately 2% of the biosynthetic energy of a bacterial cell and over 40 genes and 15 operons in *Escherichia coli* (Macnab, 1987). However the ability to sense and move around an environment in response to environmental signals can be advantageous.

1.2.2 The Flagella Regulon

The expression of flagella mediated motility is well characterised and is regulated in a hierarchical manner by up to 50 genes in set classes. The expression of genes in a given class is essential for the expression of genes in a

lower class (for reviews see Blair, 1995; Macnab, 2003). At the top of this hierarchy is the master (Class I) operon (Table 1.0).

The flagella master operon consists of *flhDC* (nomenclature taken from *E. coli* and *Salmonella*) that regulates the mainly structural genes of the class II operons encoding the basal body and associated proteins. Class II genes include the σ^{28} factor gene *fliA*, which regulates transcription of the class III genes and these include the hook, filament and chemotaxis genes necessary for a correctly functioning flagella filament. Furthermore, there are genes that are not required for flagella structure or function that are regulated by *flhDC*, or other constituents of the flagella cascade. These include the phospholipase gene *yplA* in *Yersinia enterocolitica*, which is both regulated and secreted by the flagella regulon, and constituents of the cell division regulon in *E. coli* (Schmiel *et al.*, 2000; Young *et al.*, 1999; Pruß *et al.*, 1997). The significance of the non-flagella, *flhDC* regulated genes is discussed below with regard to bacterial pathogenicity. The construction of the flagellin filament occurs external to the cell and requires an export pathway for the subunits. It appears that this export occurs through the nascent flagella structure with nearby apparatus to deliver the proteins to the channel (Macnab, 2003). This flagellar export machinery is homologous to the type III export machinery that mediate bacterial host interactions and can mediate the secretion of non-flagella related gene products (Plano *et al.*, 2001). The significance of this is also discussed below with regard to bacterial pathogenicity.

1.2.3 Motility in Bacterial-Host Associations

Typically, motility itself may be a key virulence or symbiosis factor being necessary for the location and/or invasion of the host niche by the bacterium. Once appropriately situated in its host niche a bacterial cell may then down regulate motility. For example, motility is important for *Vibrio fischeri* to form initial interactions with its host *Euprymna scolopes*, however, after colonisation of the light organ the bacterial cells appear to repress motility (Graf *et al.*, 1994; Ruby and Asato, 1993). Alternatively, in order to spread infection to other areas pathogenic bacteria may continue to express motility genes. Therefore the correct

expression of motility is essential for the correct host interactions of numerous pathogens (Otterman and Miller, 1997).

1.2.4 Motility in Pathogens

Bordetella are Gram-negative bacteria that are important pathogens as they infect the respiratory tract of a number of animals. The genus *Bordetella* includes *Bordetella pertussis*, which causes whooping cough in humans; other species are pathogens of birds, pigs and rabbits. The BvgAS two-component pathway controls all known virulence determinants in *Bordetella* and forms the basis of two phases, a Bvg^+ phase (requiring BvgAS mediated virulence genes) and a Bvg^- phase (expression of Bvg repressed genes and a lack of expression of Bvg activated genes) (Deora *et al.*, 2001). Flagella production is indicative of the Bvg^- phase and *Bordetella bronchiseptica* homologues of *flhDC* are repressed by BvgAS (Akerley *et al.*, 1995). When the *flhDC* homologues were expressed ectopically in the Bvg^+ phase the bacteria produced flagella and these cells were then defective in colonisation of the trachea in rat models (Akerley *et al.*, 1995).

Alternatively non-chemotactic and non-motile aflagellate mutants of the fish pathogen *Vibrio anguillarum* showed attenuated virulence via the natural route of infection, which is submersion in *Vibrio* containing water (O'Toole *et al.*, 1996). Furthermore the flagellin gene *flaA* is essential for full virulence; a mutation in this gene resulted in a partially motile mutant with a shortened flagellum that was attenuated for virulence both by submersion and by injection (Milton *et al.*, 1996).

Many bacterial pathogens also co-regulate motility with virulence factors, either together or inversely. The human pathogen *Yersinia enterocolitica* inversely co-regulates eukaryotic cell invasion genes and motility genes (Badger and Miller, 1998). Conversely, *Salmonella enterica* serovar Typhimurium regulates virulence with motility as the FlhDC regulated gene *fliZ*, either directly or indirectly, positively regulates the *hilA* gene, which is in turn essential for transcription of invasion genes (Iyoda *et al.*, 2001).

The expression of some *flhDC*-regulated genes can also affect host interactions in a flagella/motility independent way. The pathogen *Y. enterocolitica* produces the virulence factor phospholipase encoded by *yplA*, which is regulated by *fliA* in an *flhDC*-dependent manner. Furthermore, YplA is exported via the flagella export apparatus (Schmiel *et al.*, 2000; Young *et al.*, 1999). The flagella export apparatus bears similarities with the type III secretion systems of many pathogenic bacteria and it has been suggested that flagella and type III secretion systems may have similar origins (Macnab, 2003; Plano *et al.*, 2001). A phospholipase produced by *Serratia liquefaciens* is also regulated and secreted by products of the flagella regulon (Givskov *et al.*, 1995). Furthermore an *flhD* mutant in *Xenorhabdus* was attenuated in lipolysis, haemolysis and insect virulence, suggesting a similar role in *Xenorhabdus* for the FlhD controlled production of factors that mediate host interactions (Givaudan and Lanois, 2000). These examples demonstrate that the flagella regulon mediates the expression and secretion of gene products, which mediate host interactions in a flagella independent manner, in a wide range of bacterial pathogens.

1.2.5 Motility in Symbionts

Rhizobium, Bradyrhizobium and *Azorhizobium* can be collectively termed *Rhizobia* and are symbiotic with the leguminous plants (Paracer and Ahmadjian, 2002). The ability of these bacteria to be motile provides them with an advantage when forming symbiotic associations. In these associations motility aids contact and adsorption of bacterial cells to plant cells and increases the rate of nodule formation (Caetano-Anollés *et al.*, 1988). Therefore although motility is not essential for this symbiosis it does provide a competitive advantage.

The luminescent bacterium *Vibrio fischeri* is a symbiont of the Hawaiian bobtail squid, *Euprymna scolopes*, and the ability of this bacterium to be motile is essential for this interaction. *Vibrio* cells in the sea water encounter newly hatched *Euprymna* and are able to colonise a specialised internal organ where they bioluminesce. This bioluminescence is modulated to mimic moonlight in a strategy called counter-illumination and predators beneath *Euprymna* find it difficult to locate the squid (Visick and McFall-Ngai, 2000). The sustained colonisation of the light organ of the newly hatched *Euprymna* requires physical

changes to occur in the host and the coordinate regulation of a number of genes in *Vibrio* suggesting bacterial-host recognition and communication (Visick and McFall-Ngai 2000). Molecular techniques have allowed the characterisation of several genes necessary for this symbiosis and motility was the first bacterial phenotype to be recognised as essential for symbiotic colonisation (Graf *et al.*, 1994).

V. fischeri is a common marine micro-organism that is present in the warm seawater off the coasts of the Hawaiian islands. It has been suggested that the ventilation of the seawater by the squid partner results in the uptake of environmental *Vibrio* cells (Visick and McFall-Ngai, 2000). The *V. fischeri* cells then aggregate for several hours on host derived mucus structures in a non-specific way as polystyrene microbeads were also shown to collect in these structures (Nyholm *et al.*, 2000). The bacteria then actively migrate towards the pores of the light organ; this migration uses flagella mediated motility as normally flagellated non-motile mutants do not migrate (Graf *et al.*, 1994). Furthermore it was shown that it is the correct expression of flagella that is important in aggregation and migration as hyper flagellated and hyperswimming *V. fischeri* show slower aggregation and subsequent migration (Milikan and Ruby, 2002). Once inside the immature light organ the bacteria then enter special crypts and proliferate. During proliferation in, and occupation of, the light organ it was suggested motility was not necessary as cells appeared to lose their flagella between 12 and 48h after infection (Ruby and Asato, 1993). However, recent data suggests a more complex light organ structure including three blind-ended diverticula in the largest crypt, which house non-motile *V. fischeri*, while the main crypt space itself is occupied by larger than average, flagellated *V. fischeri* (Visick and McFall-Ngai, 2000). Interestingly this spatial segregation of flagellated and non-flagellated *V. fischeri* in structurally distinct regions suggests a role for motility in symbiosis after the initial colonisation events have occurred.

Motility and other symbiotic factors may also be co-ordinately regulated as the flagella regulon in *V. fischeri* includes several genes with no obvious role in flagella production or motility (Millikan and Ruby, 2003). Indeed a flagella transcriptional regulator mutant (*flrA*), when supplied with the gene *in trans*,

regained appropriate flagella expression and full motility, but did not regain full symbiosis. These results suggest that the appropriate regulation of *flrA*, and the putative symbiosis factors, is necessary for symbiosis.

Numerous pathogenic bacterial-host interactions require motility and the coordinate expression of motility with virulence factors (Otterman and Miller, 1997). The *V. fischeri*/*E. scolopes* association presents an interesting parallel as a well-defined symbiotic association where appropriate regulation of motility is necessary for symbiosis, and further, is likely to be regulated co-ordinately with other symbiotic factors.

1.2.6 Motility and Biofilm Formation

Biofilm formation in Gram-negative bacteria typically proceeds through set stages of initial attachment, microcolony formation and maturation and these have been shown to require cell surface appendages such as flagella and pili (O'Toole *et al.*, 2000; O'Toole and Kolter, 1998; Watnick *et al.*, 2001). Several suggestions have been made as to the function of flagella and pili in biofilm formation: flagella and chemotaxis may enable cells to swim towards a surface, flagella mediated motility may be necessary to overcome initial repulsion forces between the bacterial cell and the surface, flagella may be necessary to physically attach to a surface and flagella and/or pili mediated motility may also be necessary for spreading along a surface (O'Toole and Kolter, 1998; Pratt and Kolter, 1998; Klausen *et al.*, 2003; Lawrence *et al.*, 1987).

Biofilm formation is a complex event; highly dependent on specific strains and experimental conditions. *P. aeruginosa* PA14 flagella mutants are severely attenuated in initialising attachment to PVC when grown in minimal glucose and casamino acids media under static conditions (O'Toole and Kolter, 1998). However, *P. aeruginosa* PAO1 flagella mutants can attach and form biofilms on a glass surface when grown in citrate minimal media. In addition these PAO1 flagella mutants revert to being attenuated in biofilm formation when grown statically in glucose minimal media with casamino acids (Klausen *et al.*, 2003). These results indicate flagella are only conditionally required for biofilm formation, and biofilm formation itself is dependent on bacterial subspecies,

surface materials and nutrient signals. Further to this last conclusion Klausen *et al.*, (2003), demonstrated that wild-type *P. aeruginosa* PAO1 forms architecturally distinct biofilms dependent on the carbon source in the environment. It is apparent that for *P. aeruginosa* flagella are not essential for biofilm formation under all conditions, although they do contribute to attachment and maturation of the biofilm. A common theme thus emerges suggesting bacteria use cell surface structures in the initial stages of biofilm formation and mutants in flagella or pili genes form altered biofilms.

1.2.7 Motility in *Photorhabdus* and *Xenorhabdus*

1.2.7.1 Swimming

Initial work with primary and secondary variants of *Photorhabdus* and *Xenorhabdus* suggested links between phenotypic variation and motility; the primary variants being motile through peritrichous flagella and secondary variants non-motile due to absence of flagella (Givaudan *at al.*, 1995). However recent work by Hodgson *et al* (2003) suggests that both phenotypes are motile under appropriate conditions, i.e. the secondary variant is motile under anoxic conditions. The *Photorhabdus* infected insect cadaver at the later stages of infection is an anaerobic environment making it highly likely that the secondary variants will be motile during infection of the insect (Rosner *et al.*, 1996). Specific expression of flagella under these anaerobic *in vivo* conditions indicates motility may be important in pathogenicity. In *Xenorhabdus nematophila* an *flhD* null mutant was found to be non-motile, aflagellate and slightly attenuated in virulence (Givaudan and Lanois, 2000). However, as discussed above, the *Xenorhabdus flhD* mutant was attenuated in lipolysis and extracellular haemolysis, suggesting these products may be secreted or regulated by the flagella regulon. Furthermore in *E. coli* FlhD has been shown to be involved in other cell processes such as cell division and protein export (Pruß *et al.*, 1997; Young *et al.*, 1999). It has been suggested therefore that the flagella regulon can effect host interactions independent of motility *per se*. Consequently specific conclusions about the role of motility *per se* in the pathogenicity of *Xenorhabdus* could not be drawn.

1.2.7.2 Swarming

Swarming is a form of movement that is flagella mediated and initiated upon contact with a solid surface (Fraser and Hughes, 1999). Upon appropriate signals normal vegetative cells differentiate into elongated hyper-flagellated swarm cells. These cells form close cell-cell interactions and undergo rapid movement across solid surfaces (Hay *et al.*, 1999). Several factors are involved in the signal to differentiate into swarm cells including cell density and surface contact (Fraser and Hughes, 1999; Harshey and Matsuyama, 1994). The essential feature of swarming is hyper-flagellation and this requires the expression of *flhDC* (Young *et al.*, 1999; Eberl *et al.*, 1996). Swarming has been associated with virulence and with the regulation of specific virulence determinants in *Proteus* (Walker *et al.*, 1999; Fraser and Hughes, 1999). Furthermore, inappropriate swarming in the symbiont *Sinorhizobium meliloti* has been associated with a defect in nodulation efficiency of its plant host (Soto *et al.*, 2002). Therefore swarming has direct effects on both pathogenic and symbiotic bacterial-host interactions. Although *Xenorhabdus* and *Photorhabdus* can swarm, the potential role of swarming in symbiosis or insect virulence has yet to be elucidated (Givaudan *et al.*, 1995; Kim *et al.*, 2003).

1.2.7.3 Twitching

Twitching motility is a specific type of gliding motility and is flagella independent; it can occur between two surfaces and is mediated by the extension and retraction of type IV pili (Skerker and Berg, 2001). Type IV pili are important in cell-cell interactions, biofilm formation and pathogenisis (O'Toole and Kolter, 1998; Wall and Kaiser, 1999). It has been suggested that the extension and retraction of type IV pili may allow bacterial cells to come into close contact with host cells, while a twitching motility may allow the spreading of cells from an infected areas in a host (Wall and Kaiser, 1999). Therefore type IV pili are structures external to the bacterial cell that can mediate close bacterial host interactions and a form of motility.

1.2.8 Summary

The ability to be motile and co-ordinately regulate the synthesis and function of motility appendages can confer advantages such as the movement towards or away from environmental stimuli. Furthermore, for bacteria associated with a

host, motility can be instrumental in appropriate niche finding and host interactions. However, the presence of cell surface appendages may also represent antigenic factors that the host may recognise; therefore appropriate regulation of these appendages is important (Hayashi *et al.*, 2001). Motility and motility appendages are also important in attachment to surfaces and biofilm formation (O'Toole and Kolter, 1998). Therefore flagella, pili, swimming, swarming and twitching are likely to have important roles in the life cycles of the bacteria that possess them.

1.3 Bacterial Membranes and Lipopolysaccharides

The bacterial cell membrane is the first line of defence against a wide range of environmental stresses including extremes of pH, osmolarity, antibiotics and host mediated responses. Furthermore, the cell membrane has to facilitate the entry and exit of nutrients and waste, and accommodate the presence of external appendages that pass through the membrane such as flagella and pili (Macab, 2003; Wall and Kaiser, 1999). The survival of bacterial cells depends largely on the ability of the cells to detect and respond to a wide variety of signals in the environment. Therefore, as well as protecting the cells from any damaging changes, the bacterial membrane must also facilitate the transduction of this information to the interior of the cell where the bacterium can interpret and respond to these changes. The structure of the bacterial cell membrane and its components are therefore highly regulated.

Bacteria can be divided into two groups depending on the structure of their cell walls, Gram-positive and Gram-negative. Both groups have a cytoplasmic (inner) phospolipid based bilayer surrounded by a layer of peptidoglycan. Gram-positive bacteria have a thick peptidoglycan layer while Gram-negative have a thin layer. In Gram-negative bacteria this thin layer of peptidoglycan is then surrounded by a second lipid bilayer (Fig 1.2) the inner leaflet of which is mainly comprised of phospholipids, while the outer leaflet is mainly comprised of lipopolysaccharides

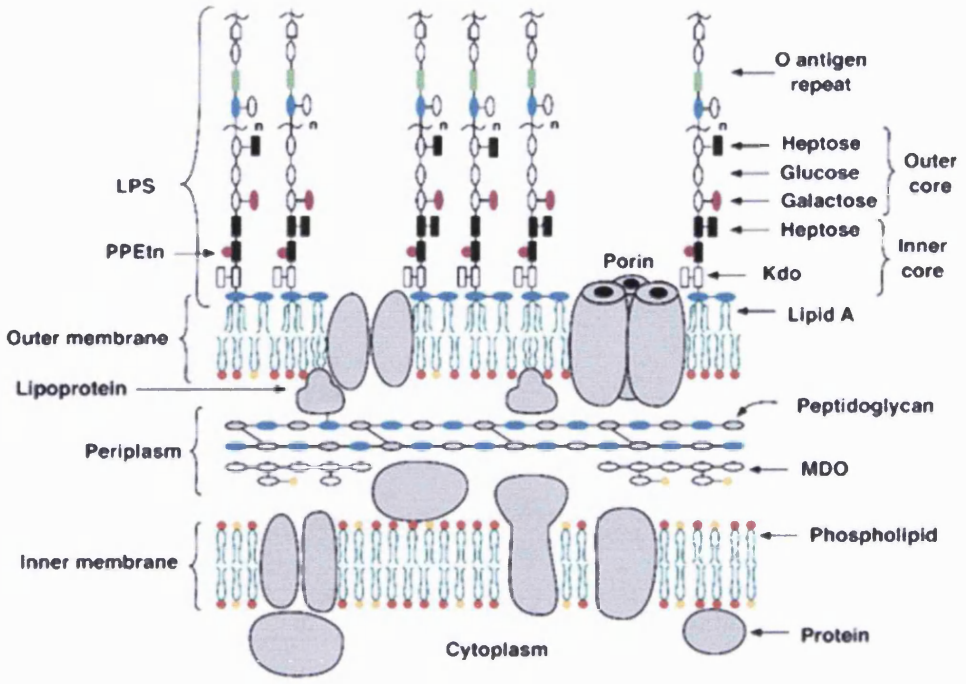


Figure 1.2. Diagram of a typical Gram-negative bacterial cell wall including, inner membrane, periplasm and outer membrane. Diagram also shows components of LPS including lipid A, core and O antigen sections. Modified from Raetz and Whitfield, (2002).

(LPS) (Campbell, 1996). This outer membrane also contains a set of characteristic proteins and, specific for the *Enterobacteriacae*, a polysaccharide called the enterobacterial common antigen (ECA) (Nikaido and Varra, 1987).

1.3.1 The Structure and Synthesis of LPS

LPS are typically composed of three domains, a hydrophobic lipid A anchored into the bacterial membrane, a 'core' oligosaccharide and a repeating 'O antigen' polysaccharide. The core and O antigen are structures external to the cell and can mediate bacterial-host interactions (Raetz and Whitfield, 2002; Campbell *et al.*, 2002). It appears the primary function of LPS in both symbionts and pathogens is protection against environmental and host mediated insults, as mutations in the LPS biosynthetic pathways results in bacterial strains that are sensitive to a wide

range of stresses (Barua, 2002; Yethon *et al.*, 2000). A second function of LPS in pathogens is the ability to impart virulence as LPS itself can act as an endotoxin to damage cells (Raetz and Whitfield, 2002; Dunphy, 1995). In symbionts, LPS may also contribute to the recognition of the symbiotic partner by the host (Campbell *et al.*, 2002). Interestingly both symbionts and pathogens have the capacity to alter their LPS once in contact with their respective hosts (Kannenberg and Carlson, 2001; Gunn *et al.*, 1998). This indicates that LPS is a dynamic structure and this property is important in host relationships. The structure of LPS can be divided into three distinct sections as discussed. Accordingly, the genes involved in the biosynthesis can also be divided into corresponding regions, lipid A with Kdo (3-deoxy-D-*manno*-oct-2-ulosonic acid), core and O antigen biosynthesis genes.

Minimally, Gram-negative bacteria require lipid A and Kdo to be viable; however the core and O antigen regions provide the bacteria with further protection against hostile environments (Raetz and Whitfield, 2002). Lipid A genes are expressed constitutively and are best characterised in *E. coli* and *Salmonella* (Fig 1.3). The starting molecule is a sugar nucleotide, which is acylated by LpxA, the pathway then catalyses further acylation and modifications including condensation to form a glucosamine dimer by LpxC, LpxD, LpxH and LpxB. Phosphate groups are then added by LpxK before Kdo is attached by WaaA (also known as KdtA). Finally, further acylation is provided by LpxL and LpxM (also known as *htrB* and *msbB*) (Raetz and Whitfield, 2002).

The *Photorhabdus* genome has been shown to contain homologues to *lpxA*, *lpxC*, *lpxD*, *lpxH*, *lpxB*, *lpxK*, and *kdtA*, but not to *lpxL* or *lpxM* (Duchaud *et al.*, 2003). The inner core of *E. coli* LPS generally consists of two heptose molecules that can be modified with phosphoryl groups including phosphoethanolamine, which increases LPS heterogeneity. However the outer core shows more structural diversity. *E. coli* and *Salmonella* have glucose as their first outer core sugar mediated by the glucosyltransferase WaaG; mutations in *waaG* result in strains lacking an outer core. Subsequent additions to this glucose can be glucose

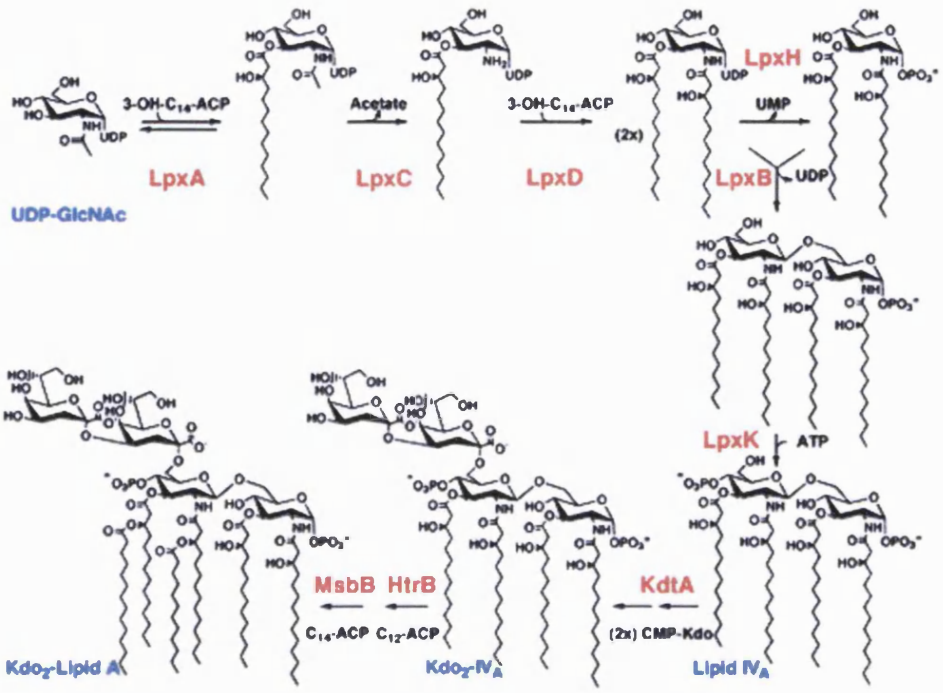


Figure 1.3. Synthesis of Kdo containing lipid A in *E. coli*. The *Photorhabdus* genome contains homologues to all the major genes involved (Duchaud *et al.*, 2003). Modified from Raetz and Whitfield, (2002).

or galactose, in a linear or branched fashion. To further diversify, some outer core regions also contain heptose. Core modifications include phosphorylation (as mentioned above) mediated by WaaP and WaaY, and the addition of a further side branch heptose mediated by WaaQ (Raetz and Whitfield, 2002). *Photorhabdus* contains several genes associated with the manufacture of the core region including the conserved genes *waaA* that transfers Kdo to lipid A and the heptosyltransferases of the inner core encoded by *waaC* and *waaF*. These genes indicate that the heptose based inner core is conserved between *Photorhabdus* and other Gram-negative bacteria. *Photorhabdus* also contains a homologue to WaaL, which ligates the core region to the O antigen (Derzelle *et al.*, 2004a). However there is significant diversity in the assigned genomic region that encodes the LPS core suggesting that the overall core structure in *Photorhabdus* is different from *E. coli* (Derzelle *et al.*, 2004a).

In *Enterobacteriacea* the synthesis of O antigen is thought to occur via three pathways each distinguished by the method of export from the inner membrane. A widespread pathway is *wzy*–dependent. The repeating O antigen unit is constructed from sugar nucleotides by glycosyltransferases and linked to a membrane bound carrier, undecaprenyl phosphate, on the cytoplasmic side of the inner membrane. It has been proposed that Wzx 'flips' the carrier and the repeat unit to the periplasmic side of the inner membrane where Wzy polymerises a nascent O antigen to the repeat unit; thus the 'newest' repeat units are located at the proximal end of the chain (Raetz and Whitfield, 2002). The product of *wzz* determines the average length of O antigen and *Salmonella* and *Shigella* have two copies that confer two different modal O antigen lengths (Murray *et al.*, 2003; Morona *et al.*, 2003). The ability of *Shigella flexneri* to manufacture two different lengths has conferred both the ability to cause pathogenicity through an outer membrane virulence protein and resist complement. It is hypothesised that the short O antigen allows the large extruding virulence protein to interact with host proteins, while other surrounding long O chains activate complement at a sufficient distance to prevent any cell damage (Morona *et al.*, 2003).

Interestingly the pathogen *Yersinia pestis* lacks O antigen (Kukkonen *et al.*, 2004). However O antigen has been shown to inhibit a membrane bound protease that causes the cleavage of plasminogen to the active protease plasmin. In addition, the same membrane protease, Pla, also degrades an inhibitor of plasminogen. This activation of plasmin causes uncontrolled proteolysis and degrades components of complement, which are factors that can increase the spread of *Yersinia* (Kukkonen *et al.*, 2004). Therefore the length of O antigen is physiologically important and likely to be regulated to a specific length as determined by the evolution of the individual bacterial strain and its interactions with its natural environment. *Photorhabdus* contains both an O antigen and a putative O antigen specific gene cluster composed of 29 genes (Derzelle *et al.*, 2004a). These include 2 homologues to *wzx* and one to *wzy*, interestingly, no homologue to *wzz* has yet been isolated. A further surface polysaccharide found in enterobacteriaceae is the enterobacterial common antigen (ECA), which consists of a trisaccharide repeating unit (Barua *et al.*, 2002). *Photorhabdus* contains a gene cluster with homology to that found in *E. coli* and *Salmonella*

suggesting *Photorhabdus* produces the conserved ECA (Derzelle *et al.*, 2004a). In conclusion *Photorhabdus* probably produces LPS with a conserved lipid A moiety, a heptose based inner core, a structurally diverse outer core and O antigen as well as synthesising the conserved ECA. Furthermore, the structure and length of the diverse O antigen is likely to be important in the interactions of *Photorhabdus* with its environments.

1.3.2 LPS and Resistance to Acid Stress

The Gram-negative outer cell wall acts as a barrier between the bacterial cell and the environment. The LPS present in the outer membrane can offer protection against a range of synthetic and host mediated antibacterial compounds (Vaara, 1992). Other host mediated environmental factors can also inhibit microbial growth e.g. a shortage in available essential nutrients such as iron, hostile aerobic/anaerobic conditions or adverse pH. However some bacteria have evolved mechanisms to adapt to and grow under these host mediated stresses. The LPS content of the outer membrane has been shown to be important in the resistance of *Salmonella* to high temperatures, high osmolarity and low pH (Thomsen *et al.*, 2003). In addition, a mutation in a *Helicobacter pylori* LPS biosynthesis gene, *wgcJ*, results in a loss of O antigen and affects the ability of this gastric pathogen to survive inorganic acidic conditions (McGowan *et al.*, 1998). Furthermore, the expression of *wgcJ* is induced upon exposure to acid indicating the importance of correct LPS synthesis in resistance of *H. pylori* to inorganic acid (McGowan *et al.*, 1998). Finally, mutations in both the ECA and LPS have been shown to affect the survival of *E. coli* when exposed to organic acid stress (Barua *et al.*, 2002). The precise role of LPS in the resistance to acid stress is unknown although several explanations have been suggested. The first is structural defence; the LPS may present a physical barrier to the influx of protons and organic acids. Secondly, LPS modifications may alter membrane permeability and surface charge to further repel these molecules. Thirdly the presence or absence of LPS may also affect the composition or function of various membrane proteins. An alteration in *E. coli* membrane protein composition occurs during growth at low pH and this has been correlated to a decrease in membrane permeability to protons (Jordan *et al.*, 1999). There are membrane proteins that act as transporters of protons either into or out of the cell

(K^+/H^+ and Na^+/H^+ antiporters) and a detrimental change in the number or function of these transporters could lead to increased susceptibility to changes in pH.

1.3.3 LPS in Resistance to Complement

A further eukaryotic defence mechanism against bacterial invaders is the complement system. This involves the binding of antibodies to a bacterial cell surface and the subsequent association of complement proteins. Initial complement proteins bind and perturb the outer membrane and subsequent complement proteins then form an attack complex and insert in the membrane resulting in cell lysis and death (Taylor, 1995; Campbell, 1996). Almost without exception Gram-negative bacteria in possession of an O antigen are more resistant to killing by complement; possibly due to physical interference of complement protein binding (Taylor, 1995; Waldor *et al.*, 1994).

Vibrio cholerae is a virulent pathogen and the causative agent of cholera. The bacteria enter the host through the oral route and colonise the small intestine where they cause diarrhoea. Mutagenesis of this pathogen yielded numerous strains that were attenuated in colonising mouse models, and many of the inserts were identified as being in LPS biosynthesis pathways, demonstrating that LPS is an important factor in *V. cholerae* virulence (Merrell *et al.*, 2002). Furthermore, studies with a *galU* mutant with an altered core showed that it was more sensitive to the complement system, cationic antimicrobial peptides, short chain organic acids and also showed colonisation defects. The authors predict that the LPS core contains no glucose and that this weakens the core-core interactions; therefore the LPS no longer provides an effective barrier against the aforementioned membrane damaging agents found in the intestine (Nesper *et al.*, 2001). Similarly, a *waaP* mutant of *Salmonella enterica* serovar Typhimurium displayed a normal O antigen but possessed an altered core. This mutant was sensitive to cationic antimicrobial peptides and was avirulent although the authors did not assess resistance to complement (Yethon *et al.*, 2000). Finally, Wzz is a cytoplasmic protein that determines the modal length of the O antigen polysaccharide. *Salmonella* has two copies of this gene wzz_{ST} and wzz_{fepE}, and the action of the protein products of these genes result in either a long or very long O

antigen repeat. Absence of both these genes results in a random but relatively short length O antigen and cells that are susceptible to complement and have attenuated oral virulence in a mouse model (Murray *et al.*, 2003).

1.3.4 LPS as Endotoxin

LPS forms the majority component of the outer leaflet of the outer membrane in many Gram-negative bacteria. LPS is considered a virulence factor in many bacterial host associations and therefore is also known as LPS endotoxin. The lipid A portion of LPS is highly toxic and injection of lipid A alone will elicit the same inflammatory response as total LPS (Lerouge and Vanderleyden, 2001). In mammalian infection models the lipid A is bound and delivered to special receptors on macrophage cells, which triggers a reaction cascade that activates factors important in clearing local infection. These include TNF-α, a tumour necrosis factor, which induces inflammation and IL1β, an interleukin, which acts on lymphocytes to cause their proliferation. However when these factors are overproduced, due to widespread sepsis, damage to the host occurs and Gram-negative septic shock follows (Raetz and Whitfield, 2002).

1.3.5 *Salmonella* LPS Modifications: PhoPQ and PmrAB in Virulence and Resistance to Antimicrobial Peptides

Bacterial pathogens are able to elicit disease by a variety of methods. Primarily they must be able to penetrate the host and respond to and survive alterations in the host environment. *Salmonella* encounters a variety of host-mediated stresses. Typically, *Salmonella* are ingested and must survive the acidic pH of the stomach before they then encounter intestinal epithelial cells to which they must adhere and invade. *Salmonella* subsequently encounter the host defences such as macrophage cells that can ingest and destroy invading bacterial cells (Groisman and Ochman, 1997). The ability to evade or survive inside macrophage cells is an important step in *Salmonella* virulence as it allows the cells to avoid the immune system. Once inside the macrophages *Salmonella* replicates and causes large vacuoles to form; mutants unable to form these vacuoles were dramatically attenuated in virulence (Lindgren *et al.*, 1996).

The two-component pathway PhoPQ is essential for virulence in *Salmonella* and regulates the expression of approximately 40 genes in response to changes (typically decreases) in levels of Mg^{2+} and Ca^{2+} (Soncini *et al.*, 1996). The levels of these cations can be an indication of intra or extracellular location, which can therefore ensure certain survival and virulence genes are only expressed in the appropriate environment (Groisman, 2001). PhoPQ is likely to be active inside macrophages, under conditions of low Mg^{2+}, where it controls the transcription of genes important for survival. Furthermore PhoPQ negatively regulates cell invasion through the transcriptional regulator *hilA*, and is therefore important in the correct temporal expression of virulence genes (Behlau and Miller, 1993; Bajaj *et al.*, 1996).

The two-component pathway PhoPQ also regulates the activation of a second two-component pathway PmrA (the response regulator) and PmrB (the sensor kinase) through the action of an intermediate protein, PmrD, which affects the activity of PmrA (Gunn and Miller, 1996; Kox *et al.*, 2000). Ferric iron and acidic pH can also activate PmrAB independently of PhoPQ (Soncini and Groisman, 1996) indicating that the PmrAB regulated genes may be important under a variety of environmental conditions.

The response regulator PmrA regulates the transcription of several genes necessary for the modification of LPS (Gunn *et al.*, 2000, Baker *et al.*, 1999) including *pmrE*, previously *pagA* or *ugd*, and *pmrHFIJKLM*. Furthermore, PmrA also regulates its own posttranscriptional activator PmrD and the transcription of *pmrG*, although the role this gene has is not yet understood. The gene *pmrE* encodes a UDP-glucose dehydrogenase, which acts on UDP-glucose in the metabolic pool converting it to UDP-glucuronic acid. This product is then the substrate for the gene products of the *pmrFHIJLKM* operon, which are responsible for the biosynthesis and ligation of 4-amino-4-deoxy-$_L$- arabinose ($_L$-Ara4N) to the 4' phosphate group on the lipid A of LPS (Baker *et al.*, 1999; Trent *et al.*, 2001). This addition changes the electrostatic interactions of the cell; the amino group of $_L$-Ara4N is positively charged and the addition of $_L$-Ara4N to lipid A reduces the overall negative charge of LPS, which can reduce the attraction of positively charged molecules.

LPS is negatively charged through phosphoryl groups on the lipid A and core region and tight LPS-LPS interactions are maintained through cross-linking of LPS with divalent cations (Raetz and Whitfeild, 2002; Vaara, 1992). These phosphoryl groups are essential for stable LPS-LPS interactions and membrane structure and therefore they help form an effective barrier against agents that act on membranes. The *waaP* gene product has been shown to be responsible for phosphorylation of the core region and a *Salmonella waaP* mutant was sensitive to membrane permeabilising agents despite containing a full O antigen (Yethon *et al.*, 2000). However, the negative charges of LPS can facilitate the interaction of detrimental positively charged agents such as cationic antimicrobial peptides. Antimicrobial peptides are a biologically important group of molecules produced by hosts in response to infection by microbes. Insects produce a variety of antibacterial peptides as part of the innate immune response, these are secreted into the haemolymph and include (depending on insect species) attacins, defensins, lysozyme and cecropins (Gillespie and Kanost, 1997). Vertebrates also typically produce small polycationic antimicrobial peptides that act on bacterial membranes and these can be divided into 4 main groups, defensins, proline and arginine-rich peptides, amphipathic helical peptides and a fourth group comprising peptides found in the skin of one specific frog family (Nicolas and Mor, 1995). Defensins are found in the circulating mammalian neutrophil and macrophage cells and mammalian neutrophils also produce the proline and arginine rich family of antimicrobial peptides (Nicolas and Mor, 1995). Therefore a mammalian pathogen such as *Salmonella* would encounter a variety of antimicrobial peptides especially when invading macrophage cells and would need to be able to adapt and overcome this host defence. As the action of antimicrobial peptides is based on their positive charge and membrane damaging action, defence against this has led to bacterial membrane modifications.

Polymyxin B is a positively charged, antimicrobial peptide of bacterial origin and is able to bind to the LPS of Gram-negative bacteria and consequently permeabilise the outer membrane. This provides polymyxin B with access to the inner membrane where it causes leakage of cytoplasmic components and cell death (Vaara, 1992). The change in membrane charge due to the addition of the

positively charged $_L$-Ara4N reduces the affinity of some cationic antimicrobial peptides for LPS and therefore confers resistance (Gunn *et al.*, 2000). In support of this, LPS from constitutively active *pmrA* mutants of *Salmonella* have been shown to contain 4-6 times more $_L$-Ara4N on their lipid A, and bind 50% less polymyxin than the LPS from the parent strain (Vaara *et al.*, 1981).

Further membrane permeabilising agents include polylysine and protamine, chelators such as EDTA that remove the stabilising divalent cations, Tris, which may displace the cations and excessive Na^+ which may also replace the cations (Vaara, 1992). Disruption of the strong lateral interactions between LPS molecules is thought to result in the release and loss of LPS. Subsequently phospholipids from the inner leaflet migrate to the outer leaflet to form patches of phospholipid bilayers. It is these phospholipid islands that may allow interaction and diffusion of normally repelled antimicrobial peptides (Vaara, 1992). Polymyxin resistance conferred by a mutation in *pmrA* in *Salmonella* also confers cross-resistance to EDTA, polylysine and protamine. Furthermore, an *E. coli* mutant resistant to polymyxin was also resistant to ampicillin, penicillin, gentamicin, kanamycin and numerous other antibiotics (Rahaman *et al.*, 1998). Moreover, sublethal concentrations of polymyxin render *E. coli* and *Salmonella* susceptible to other antibacterial agents probably through the action of disrupting the outer membrane (Vaara, 1992). Therefore, resistance to cationic antimicrobial peptides is important in pathogens of mammals and insects and can provide cross-protection to other host mediated stresses.

1.3.6 Further Defence Mechanisms Against Antimicrobial Peptides in *Salmonella*

Alternative modifications to LPS that induce resistance to antimicrobial peptides include the addition of palmitate, a fatty acid, into lipid A. This modification is dependent on the PhoP regulated gene *pagP* that encodes a palmitoyl transferase (Bishop, 2000). *Photorhabdus* has a gene (*plu2784*) with 59% identity at the amino acid level with the product of the *Salmonella pagP* gene suggesting *Photorhabdus* may be able to make a similar lipid A modification. Furthermore Derzelle *et al.*, (2004a) have shown that *pagP* in *Photorhabdus* is regulated in a Mg^{2+} dependent manner.

It has been suggested that the fatty acid modification induced by PagP may increase the hydrophobic interactions of the fatty acid tails, thus reducing fluidity and stabilising the membrane (Guo *et al.*, 1998). Furthermore, in addition to the $_{L}$-Ara4N modifications of lipid A, the LPS of constitutively active *pmrA* mutants contained higher levels of phosphoethanolamine. This modification may contribute to antimicrobial peptide resistance and is also induced by PmrA activated genes (Vaara *et al.*, 1981; Gunn *et al.*, 1998). The addition of phosphoethanolamine can occur on phosphate groups in the lipid A moiety and the core region and may further mask negative charges. The presence of phosphoethanolamine may even negate the requirement for $_{L}$-Ara4N (Gunn *et al.*, 1998).

The gene *pgtE* encodes an outer membrane protease, which is under the control of PhoPQ and cleaves antimicrobial peptides with an alpha helical structure and is likely to contribute towards resistance in synergism with other mechanisms (Guina *et al.*, 2000). *Photorhabdus* does not appear to have a homologue of this gene. Lastly the gene *mig-14* has been shown to contribute to the resistance of *Salmonella* to antimicrobial peptides and is under the partial control of PhoP (Brodsky *et al.*, 2002). However, no modification in LPS could be detected in a *mig*-14 mutant strain and the sequence of the gene suggests it may have homology to a family of transcriptional regulatory proteins involved in stress responses. *Photorhabdus* has a gene (*plu0499*) with 34% identity at the amino acid level with the product of the *Salmonella mig*-14 gene suggesting that *Photorhabdus* may have a similar unknown second system for protection against antimicrobial peptides. The precise nature of the role of *mig-14* in resistance to antimicrobial peptides has yet to be elucidated and highlights the possibility of LPS independent mechanisms of resistance (Brodsky *et al.*, 2002).

Therefore the resistance to antimicrobial peptides is induced via a variety of systems including both PhoPQ and PmrAB dependent and independent, although the PhoP dependent systems are most common. This indicates PhoP is a master regulator of cationic antimicrobial peptide resistance primarily through modifications of the outer membrane and LPS, which either mask or remove

negative charges. Furthermore these modifications that induce resistance contribute towards survival of host-mediated stresses.

1.3.7 PhoP in *Photorhabdus*

Recently the role of the PhoPQ two-component pathway was examined in *Photorhabdus*. A mutation in *phoP* was made and the strain was shown to be avirulent and more sensitive to antimicrobial peptides (Derzelle *et al.*, 2004a). PhoPQ has been shown to be important for intracellular survival for a number of pathogens including *Salmonella*, *Shigella* and *Yersinia*, but it is not known yet whether *Photorhabdus* has an intracellular phase during infection (Groisman, 2001; Oyston *et al.*, 2000). However, PhoPQ homologues are important for the full virulence of *E. carotovora*, a Gram-negative plant pathogenic bacterium, which is also not known to invade plant cells (Flego *et al.*, 2000). The PhoPQ pathway in *Photorhabdus* does not appear to be essential for Mg^{2+} uptake, as a *phoP* mutant is able to grow in low Mg^{2+} conditions. Furthermore, there are several amino acid substitutions in the extracellular sensor domain of PhoQ, specifically, in the area homologous to that which binds the divalent cations Mg^{2+} and Ca^{2+} in *E. coli* suggesting divergent roles for the PhoPQ system in *Photorhabdus*. However, PhoP did appear to mediate changes in LPS in a Mg^{2+}-dependent manner despite the fact that PmrAB does not appear to be conserved in *Photorhabdus* (Derzelle *et al.*, 2004a). The presence of *Photorhabdus* in the insect haemolymph and the knowledge that *Photorhabdus* contains PhoP-regulated *pmrHFIJLKM* homologues suggests that LPS modifications may be important in the resistance of *Photorhabdus* to antimicrobial peptides present in the insect haemolymph. Further to this, the *phoP* mutant was shown to be more susceptible to cecropins and polymyxin *in vitro* (Derzelle *et al.*, 2004a).

These results suggest that while the PhoPQ two-component pathway appears to be conserved in *Photorhabdus* several salient differences in the primary function of this pathway are apparent. The changes in the ligand-binding domain, the non-essential nature of PhoPQ in a Mg^{2+}-limiting environment and the possibility that *Photorhabdus* is an extracellular pathogen suggest that PhoPQ and its regulated genes may have novel roles in virulence and survival of this entomopathogen.

1.3.8 LPS and Motility

Swarming is a form of surface translocation and typically requires signals such as surface contact and cell density (Fraser and Hughes, 1999). Swarming bacteria are generally encased in slime, a mixture of carbohydrates, proteins, peptides and surfactants, which provides a milieu that aids motility by reducing or overcoming surface tension (Toguchi *et al.*, 2000). Surfactants are required for swarming in *Serratia* and serve to reduce surface tension of the media and thus contribute towards translocation and for *Serratia liquefaciens* this biosurfactant is a lipopeptide (Lidnum *et al.*, 1998). LPS has been implicated as critical for the swarming phenotype of *Salmonella* as a large number of transposon mutants with defects in swarming were identified as being in LPS biosynthesis genes (Toguchi *et al.*, 2000). Furthermore, gene expression profiles demonstrated LPS genes were significantly induced in response to a solid surface (Wang *et al.*, 2004). Possible roles for LPS in swarming have been suggested and these include increasing the hydration of the cell surface by the presence of hydrophilic O antigen and the possibility that LPS may form a major constituent of slime (Toguchi *et al.*, 2000; Wang *et al.*, 2004). A *waaG* core modification mutant was precocious for swarming and manufactured excessive slime; probably through induction of the two-component pathway RcsB and RcsC (Parker *et al.*, 1992).

Interestingly, LPS modifications have also been shown to affect swarming migration of *Proteus mirabilis*. A *Proteus* mutant that lacked the $_{L}$-Ara4N modification of the lipid A moiety was sensitive to antimicrobial peptides and was unable to swarm (McCoy *et al.*, 2001). Furthermore a *Salmonella* mutant disrupted in the gene *pmrK*, previously identified as being essential for $_{L}$-Ara4N biosynthesis and addition to lipid A, was also unable to swarm (Kim *et al.*, 2003). A possible explanation is that the modification of lipid A with $_{L}$-Ara4N changes the surface charge and hydrophobicity of the cell; therefore affecting cell-to-cell or cell-surface interactions necessary for swarming. The LPS O antigen is also important in the social gliding (twitching) motility of *Myxococcus xanthus* as four independent LPS biosynthesis mutants were defective in translocation. Once again the authors suggest that LPS acts as a mediator of the cell-to-cell or cell-surface interactions necessary for this type of movement (Bowden and Kaplan, 1998). These results strongly suggest LPS has a significant

role in the motility of bacteria and can effect the interactions of the bacterial cells with the environment around them.

1.3.9 LPS in Symbiosis

Rhizobia are Gram-negative bacteria that live in the soil and form symbiotic associations with the roots of leguminous plants where they fix atmospheric nitrogen and supply it to the plant. In return the plant provides the bacteria with the large amount of ATP and an anaerobic environment needed to reduce nitrogen (Paracer and Ahmadjian, 2002). The process of symbiosis involves signalling molecules from plant and bacterium as well as structural changes in both organisms. Briefly, the roots of the legume secrete flavonoid molecules, which induce the transcription of *nod* (nodulation) genes in the bacterium. These *nod* genes encode nodulation factors, lipo-oligosaccharides, which are then released back into the environment where they cause the root hair to respond by curling and cell division in the root cortex. Furthermore it is the presence of particular *nod* genes that confer host range specificity (Fisher and Long, 1992). The bacteria penetrate the root hair and travel down an infection thread towards the dividing cortical cells that become nodules. Once released from the infection thread into these cells the bacterial cells become bacteroids, which are extremely large (40 time the size of free-living cells) non-motile cells with thin walls to allow passage of nutrients back and forth to the plant cells (Paracer and Ahmadjian, 2002). The role of LPS in the initiation of contact, maintenance of infection thread, infection of nodules and long-term survival in bacteroids is discussed below.

LPS or other specific cell surface components do not appear to be essential in initial bacterial-plant contact or bacterial aggregation, although motility has been shown to confer an advantage (Kannenberg and Brewin, 1994; Caetano-Anollés *et al.*, 1988). However, the close host contacts during infection thread and bacteroid development suggests that bacterial cell surface appendages may be involved in mediating cell-cell attachment or recognition. *Rhizobium* mutants with defects in LPS are attenuated in normal symbiosis (Noel *et al.*, 1986). Furthermore, *Rhizobium* with defective LPS elicited localised plant defence reactions suggesting that the lack of LPS means the bacterial cells are not

recognised as symbionts and are attacked as if they are pathogenic invaders (Perotto *et al.*, 1994).

Uncharacterised *Rhizobium* mutants that appeared to lack LPS were identified as initiating infection threads with abnormal morphology that stopped development prematurely (Noel *et al.*, 1986). Characterised *lpsB* mutants of *Sinorhizobium meliloti* possessed a smooth O antigen but showed an altered LPS core composition, and although they could generate nodules and invade normally, they failed to establish chronic intracellular infection (Campbell *et al.*, 2002). In addition, these mutants were sensitive to cationic antimicrobial peptides and the authors suggested that the mutant might not establish chronic infection due to sensitivity to innate plant antimicrobial peptides that may be present in the nodules.

Recent studies have further highlighted the importance of LPS to bacteroids. Sindhu *et al.*, (1990) demonstrated that LPS isolated from free-living cells showed structural differences to LPS isolated from bacteroids. Moreover recent studies have shown that modifications in bacterial LPS can be induced by environmental conditions similar to those found in nodules, such as low O_2, and that these LPS are hydrophobic and resemble LPS isolated from bacteroids (Kannenberg and Carlson, 2001). Fraysee *et al.*, (2003) suggested that hydrophobic LPS might enable the bacterial cells to form the close interactions with the hydrophobic plant cell walls and membranes necessary for metabolic exchange. These results suggest that the structure of LPS is dynamic and the bacteria synthesise different LPS in response to different environmental cues.

The life cycle and infection process of *Rhizobia* involve distinct stages, survival in the soil, attachment to root hairs, survival and passage through the infection thread and finally passage into, and survival in, the nodule as a bacteroid. The ability to alter LPS may facilitate the different interactions needed by altering properties such as cell surface hydrophobicity and consequently attachment as well as resistance to environmental and host stresses.

In summary four basic roles of LPS in symbiotic plant interactions have been suggested. i) LPS may act as a recognition signal, either promoting symbiotic interactions or suppressing a pathogenic response. ii) LPS may facilitate physical attachment to host cells (Fraysee *et al.*, 2003). iii) An intact LPS may be structurally important in maintaining a non-leaky cell wall; therefore preventing the release of bacterial metabolites that may elicit a negative response and, synergistically, a complete O antigen may mask the normally endotoxic lipid A moiety (Kannenberg and Brewin, 1994). iv) Intact LPS may enable the cell to resist host-induced stresses (Campbell *et al.*, 2002). Moreover the ability to alter LPS in response to differing environments may be an important feature in adapting to life as an endosymbiont.

1.3.10.1 LPS in *Photorhabdus* and *Xenorhabdus*

Both *Photorhabdus* and *Xenorhabdus* contain smooth LPS in their outer membrane (Dunphy and Webster, 1988; Dunphy, 1995; Clarke, 1993). The presence of LPS may affect the interaction of *Photorhabdus* with the insect immune response. The response of an insect to invading bacteria involves both cellular and humoral immune responses. These include activation of haemocytes such as plasmatocytes, which phagocytose the bacterial cells, nodule formation, encapsulation, activation of phenoloxidase and the production of antimicrobial agents such as lysozyme and antimicrobial peptides such as the cecropins (Gillespie *et al.*, 1997). The role of LPS in the interactions with these insect responses is discussed below.

1.3.10.2 LPS and Cellular Responses

After infection into the insect haemolymph *Photorhabdus* and *Xenorhabdus* rapidly grow and divide and the insect generally dies within 48h (Akhurst and Dunphy, 1993). In order to survive in the insect haemolypmh the bacteria must evade or overcome the host immune response. Phagocytosis is the initial response to invasion of bacterial cells, which is supplemented by nodulation upon increasing numbers of bacterial cells. Nodulation is the formation of multicellular haemocyte aggregates (nodules) that entrap bacterial cells and remove them from the haemolymph by adhering to tissues (Gillespie and Kanost, 1997). However, *Photorhabdus* is able to proliferate within the nodules of

Galleria mellonella and release LPS. This damages the cells and initiates haemocytes lysis; the bacteria are then able to re-emerge into the haemolymph (Dunphy and Webster, 1988). The release of *Photorhabdus* LPS has been shown to be directly related to damaged haemocytes *in vivo* and the emergence of cells into the haemolymph is itself parallel to increases in haemocyte damage; therefore LPS has been suggested as a virulence factor (Dunphy, 1995). The LPS, more specifically the lipid A moiety, of *Xenorhabdus nematophila* is also haemocytoxic (Dunphy and Webster, 1988) and has been shown to damage haemocytes in *G. mellonella*. Furthermore, a study with avirulent transpositional mutants of *Xenorhabdus* showed that the mutant with the lowest rate of LPS release was removed efficiently from the haemolymph and was the last to re-emerge; possibly due to a lower level of haemocyte damage (Dunphy and Hurlbert, 1995). Thus LPS is important in overcoming insect cellular responses.

1.3.10.3 LPS and Innate Responses

Humoral defence factors in larval serum with proteolytic, lysozyme and carbohydrase activity alter the outer membrane of *X. nematophila* (Dunphy and Webster, 1991). It may be these interactions and modifications that trigger the release of *X. nematophila* LPS and cause subsequent haemocyte damage (Dunphy and Webster, 1988).

The insects defence system includes the enzyme phenoloxidase, which converts tyrosine to dihydroxyphenylalanine, which in turn binds to the bacterial cell surface and facilitates the removal of bacterial cells through melanization and possible opsonization (Forst and Nealson, 1996). LPS from both *X. nematophila* and *P. luminescens* can prevent the conversion of prophenoloxidase to phenoloxidase in *G. mellonella* (Dunphy and Webster, 1991; Dunphy, 1995). Therefore LPS is also important in contributing to resistance to insect innate immunity.

The presence of bacteria in the haemolymph also induces the production of antimicrobial peptides such as cecropins, peptides of approximately 4kDa (Gillespie *et al.*, 1997). Correct LPS structure is important in the resistance of bacteria to antimicrobial peptides (Gunn *et al.*, 2000). Furthermore a

Photorhabdus PhoP mutant was unable to modify its LPS, was susceptible to the insect antimicrobial peptides cecropin A and B and was avirulent *in vivo*; demonstrating the importance of correct LPS structure in pathogenicity (Derzelle *et al.*, 2004a).

LPS from *Photorhabdus* and *Xenorhabdus* therefore has a significant role in the pathogenicity of insect hosts and in the defence against both the cellular and humoral insect immune response. However the role of isolated LPS from *Photorhabdus* has been investigated and purified LPS was found not to be lethal (Clarke and Dowds, 1995). This suggests LPS may be more protective for the *Photorhabdus* cells than toxic for the insect. Nonetheless LPS clearly contributes to the epidemiology of pathogenicity of both *Xenorhabdus* and *Photorhabdus*.

1.3.11 Summary.

For Gram-negative bacteria the possession of LPS can confer significant advantages as LPS constitutes a barrier to numerous harmful substances including many host mediated substances and environments (Vaara, 1992). Furthermore, the ability of some bacteria to modify LPS allows for adaptation to specific environmental niches and LPS can aid both pathogens and symbionts in surviving host-mediated stresses. LPS also represents a signature for bacteria that can help symbiotic bacteria be recognised by their hosts. For some pathogenic bacteria LPS contributes towards epidemiology of pathogenicity inducing inflammation and septic shock or damaging host cells. However the recognition of lipid A as an indication of the presence of bacteria can also contribute towards the host clearing infection and overcoming the bacterial invaders. Furthermore O antigen containing LPS can hinder the action of membrane associated virulence proteins. Therefore the presence of LPS, containing lipid A, core and O antigen moieties can confer advantages; however they represent recognisable signatures and for pathogens this may be detrimental.

1.3.12 Aims and Objectives

Bacteria interact with their host environment and respond to signals and changes in it. These responses include changes in their gene expression, which results in altered host interactions either leading to pathogenic or symbiotic associations.

The aim of this study is to elucidate the mechanisms of host interactions involved in *P. luminescens* symbiosis and pathogenicity.

As secondary variant *P. luminescens* are equally pathogenic towards insects yet non-symbiotic with their nematode partners it is assumed the phenotypic differences between the primary and secondary variants are important for symbiosis. One of the many phenotypic differences is that secondary variant *P. luminescens* are non-motile, together with other evidence for the importance of motility in host interactions, the role of motility in this model system was investigated. The objective was to isolate motility mutants and investigate the role of motility in the interactions of *P. luminescens.*

CHAPTER 2

2.0 Materials and Methods.

2.1 Strains, Plasmids and Growth Conditions

Unless otherwise stated, 'TT01' refers to rifampicin resistant *Photorhabdus luminescens* subsp *laumondii* TT01. Strains and plasmids used in this study are listed in table 2.0. *P. luminescens* strains were grown at 28°C and *E. coli* strains at 37°C unless otherwise stated and liquid cultures were shaken at 200rpm. Media was supplemented with appropriate antibiotics at the following concentrations, kanamycin 30μg/μl, ampicillin 100μg/μl, tetracycline 15μg/ml rifampicin 50μg/μl. Strains were maintained as glycerol stocks (500μl 40%v/v glycerol to 500μl overnight culture) and kept at -80°C. The different media and compositions for agar plates used in this study are listed in table 2.1.

2.2 Media, Kits and Enzymes

Liquid culturing was carried out using Luria-Bertani (LB) medium (Merck) (1% peptone from casein, 0.5% yeast extract, and 1% sodium chloride in water) for culturing on solid media 15g agar (Merck) per litre were added. Plasmids were purified using Wizard Plus Miniprep kits from Promega. PCR reactions were cleaned using a Stratagene kit. Enzymes were obtained from New England Biolabs or Promega.

2.3 Overnight Starter Cultures

A single colony of the required bacterial strain was inoculated into a 14ml round bottom polypropylene Falcon tube containing 3ml LB, supplemented with appropriate antibiotics, using a sterile loop. The culture was incubated in a shaking water bath at the appropriate temperature overnight.

2.3.1 Polymyxin Resistance Assay

Standard overnight cultures of the *P. luminescens* strain of interest were grown. Fresh 13ml Falcon tubes were prepared containing 3ml of sterile LB and

Species, Strains and plasmids		Genotype	Source
Photorhabdus luminescens	TT01	Wild type	Lab stock
	TT01 Rif	Rifampicin resistant	This study
	BMM316	TT01 Rif,-*flgG*::Km^r	This study
	BMM305	TT01 Rif,-*pbgE1*::Km^r	This study
Escherichia coli	CC118 (λ*pir*)	Δ*(ara-leu) araD* Δ*lacX74 galE GlaK phoA20 thi-1 rps rpo argE(*Am*) recA1*	(Herrero *et al* 1990)
	JM109	F' *traD36 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*) *M15*/ Δ(*lac-proAB*) *glnV44 e14*$^-$ *gyrA96* recA1 relA1 endA1 thi hsdR17	Lab stock
Plasmids	pBR322	Ap^r, Tc^r. Cloning vector	Lab stock
	pTRC99a	Ap^r, Tc^r. Cloning vector	Lab stock
	pUTkm	Ap^r; Tn*5*-based delivery plasmid with Km^r	(Herrero *et al* 1990)
	pLOF	Ap^r ; Tn*10*-based delivery plasmid with Km^r	(Herrero *et al* 1990)

Table 2.0. List of strains and plasmids used in this study.

appropriate antibiotics. Polymyxin sulphate was dissolved in PBS and added to the required concentration, finally, 20μl of the overnight culture were inoculated into the fresh overnight tubes. The cultures were incubated in a shaking water bath at 28°C overnight and the OD_{600} taken. Overnight growth was described as relative to parental strain without polymyxin (Relative growth of 1).

2.3.2 Organic and Inorganic Acid Tolerance

Standard overnight cultures of the *P. luminescens* strain of interest were grown. For inorganic tolerance, LB was supplemented with 50mM MES and the pH was adjusted to different concentrations using concentrated HCl or NaOH and autoclaved. For organic acid tolerance, LB was supplemented with 50mM MES and different concentrations of organic acid added. The pH of this buffered media was adjusted to either 7 or 6.5 before being autoclaved, then 3ml of this sterile LB containing buffered organic or inorganic acid were pipetted into fresh

Agar	Composition per litre dH_2O	Use
Luria Burtani (LB) agar	10g tryptone, 5g yeast extract, 10g NaCl (Merck) 15g agar (Merck)	Normal culture
Swim agar	10g tryptone, 5g yeast extract, 10g NaCl, (Merck) 3g agar (Merck)	Motility Assay
Lipase agar	28g nutrient agar (Oxoid), 0.5% (v/v) Tween 80 (BDH)	Lipase Assay
Lipid agar	5g Yeast extract (Difco), 5ml cod liver oil (Seven seas), 25g nutrient agar (oxoid), 10g corn syrup (Karo), 2g MgC $l_2.6H_2O$ (Fischer)	Nematode growth and development
NBTA	28g nutrient agar (Oxoid), 25mg Bromothymol blue (USB), 30mg, 2,3,5,TTC	Dye uptake
MacConkey agar	Merck	Dye uptake
EMB	(Eosin mythyl blue agar) Oxoid	Dye uptake
CAS agar	60.5mg chromo azurol sulphate (CAS) in50ml sterile water, 10ml iron III sulphate solution (1mM $FeCl_3.6H_2O$, 10mM HCl). 72.9mg HDTMA dissolved in 40ml sterile water. (100ml Total)	Siderophore assay

Table 2.1. List of agars, their composition and suppliers used in this study.

13ml Falcon tubes with the appropriate antibiotics. Finally, 20µl of the overnight culture were inoculated into the fresh overnight tubes; the cultures were incubated in a shaking water bath at 28°C overnight and the OD_{600} taken. Overnight growth was described as relative to parental strain without acid (Relative growth of 1).

2.4 Phenotypic Analysis

The *Photorhabdus* strain of interest was grown overnight as described, resuspended to an OD_{600} of 1.0 and 3µl of this culture were then pipetted onto the following agars, and incubated for 48h at 28°C, for plate based phenotypic assays. LB agar was used to determine pigment production and bioluminescence (bioluminescence was assessed on a Fujifilm Intelligent Dark Box II). EMB, NBTA and MacConkey agar were used to determine dye uptake. Lipase indicator agar was used to determine lipase production (a precipitate forming around the *Photorhabdus* colonies indicated lipase production). CAS agar was made (60.5mg chromo azurol sulphate (CAS) were dissolved in 50ml sterile water, 10ml iron III sulphate solution (1mM $FeCl_3.6H_2O$, 10mM HCl) was added. This solution was then slowly added with stirring to 72.9mg HDTMA dissolved in 40ml sterile water. The resulting dark blue liquid was then autoclaved. This CAS solution was added in a ratio of 1:10 to stock LB agar) and this media was used to determine the production of siderophores, which results in the appearance of yellowing zones around the *Photorhabdus* colonies.

2.4.1 Protease Gels

The *Photorhabdus* strain of interest was grown for 48h, 2ml of culture were pelleted and the supernatant retained and filter sterilised. Equal ODs were loaded without boiling onto a protease gel. Briefly, 0.1% gelatin was added to a standard 12.5% SDS resolving gel and heated to dissolve before cooling and adding APS and TEMED, a standard 5% SDS stacking gel was used. The gel was washed in 0.1M glycine, 2.5% Triton X-100 for 30mins (x2), and was finally left overnight before staining with Coomassie blue stain. The appearance of clear bands on a blue background indicated protease production.

2.4.2 Catalase Production

The *Photorhabdus* strain of interest was grown overnight as described and resuspended to an OD_{600} of 1.0, 10µl of this suspension were pipetted onto a glass slide and 10µl 3% hydrogen peroxide were then pipetted directly onto the suspension. Small bubbles and fizzing indicated catalase production.

2.4.3 Antibiotic Production

The *Photorhabdus* strain of interest was grown overnight as described and resuspended to an OD_{600} of 1.0, 5µl were pipetted onto LB agar and incubated at 28°C for 48h. The plate was overlaid with warm soft agar (0.7% agar) containing 2ml of an overnight culture of *Micrococcus luteus* per 200ml agar. The plate was then incubated at 37°C for 48h; a clear zone of no growth around *Photorhabdus* colonies indicated antibiotic production.

2.4.4 Crystalline Inclusion Proteins

The *Photorhabdus* strain of interest was grown overnight as described and diluted to OD_{600} of 0.1. Approximately 1µl of this dilution was then pipetted onto a microscope slide and the bacterial cells were observed at X1000 magnification using phase-contrast microscopy. The presence of crystalline inclusion proteins could then be visualised.

2.4.5 Biofilm Formation

Overnight starter cultures of the bacterial strain of interest were resuspended to 1.0 OD_{600} and 100µl were added to 900µl LB dispensed into 5ml culture tubes. The culture tube and briefly vortexed, the tube was then statically incubated at 28°C. After the required amount of time the OD_{600} of the culture was taken, the bacterial suspension removed and 100µl of a 1% (w/v) crystal violet solution was added, vortexed and removed. The tube was then gently washed out with water 3 times and the resulting band qualitatively analysed.

2.5 Virulence of *P. luminescens*

Galleria mellonella were purchased from Livefood UK and stored at room temperature. The *P. luminescens* strains to be injected were grown overnight as described. The OD_{600} of the culture was taken, the cell number was calculated ($2x10^8$ cell/ml = 1.0 OD_{600}) and adjusted using PBS as a dilutant. The required numbers of cells were then directly injected into the insect hemocoel using a Hamilton syringe. The injection was repeated with 10 different *Galleria* for each strain used. The insects were incubated at 25°C and monitored over 48h. Virulence was assayed using LT_{50} and LD_{50} measurements. The LT_{50} is the time

at which 50% of the insects die using a standard cell dosage. The LD_{50} is the cell dose at which 50% of the insects die at a standard time. Control insects were injected with 10µl PBS.

2.5.1 Virulence Competition

The two *P. luminescens* strains to be competed were grown overnight as described and the OD_{600} was taken for both strains, the suspensions were diluted in PBS to give approximately 10 cells/µl and mixed together in equal volumes. The mixed suspension was then directly injected into the insect hemocoel by Hamilton syringe. The insects injected were incubated at 25°C and monitored over 48h. At time of death the insects were surface sterilised by dipping in ethanol and passing through a Bunsen flame. The insect cadaver was placed in a Petri dish containing 10ml LB broth and dissected to release *Photorhabdus* cells. 2mls of this suspension were passed through sterile muslin and diluted into 8ml LB. A serial dilution of this suspension was made and appropriate dilutions were plated out onto selective media to differentiate between the two bacterial strains.

2.5.2 Virulence of the Nematode-Bacteria Complex

For the natural infections two round sheets of Whatman filter paper, one placed in the lid of a 9cm Petri dish the other in the bottom, were wet with an average of 500 nematodes in PBS, 10 *Galleria mellonella* were placed on the Whatman paper and incubated at 25°C, insect death was then monitored.

2.6 Recovery of Nematodes

Nematodes were recovered from the infected *G. mellonella* cadavers by White traps (White, 1927). These are constructed in 90mm Petri dishes by positioning a concave platform in the centre of the Petri dish. A round sheet of filter paper (Whatman No. 1) covers the platform and dips into a moat of PBS to ensure continual moistening of the paper. The cadavers are placed on top of the small platform and then incubated at 25°C. The IJs leave the cadaver and migrate down the moist filter paper into the PBS.

2.7 Growth and Development of *Heterorhabditis* on *P. luminescens*

P. luminescens strains of interest were grown overnight as described and 50µl were spread onto lipid agar in the form of a large 'Z' and incubated for 48h at 28°C. Approximately 20 surface sterile *H. bacteriophora* TT01 IJ nematodes were added and the plates were further incubated at 25°C. Development and reproduction of the nematodes was monitored over a period of weeks using a stereo microscope. IJs were collected from the lid of the Petri dish by washing with PBS and stored at 4°C for further experiments.

2.7.1 Symbiosis Competition

The two *P. luminescens* strains to be competed were grown overnight as described and the OD_{600} was taken for both strains, the suspensions were diluted in LB to give approximately 1.0 OD_{600} and mixed together. 50µl were spread onto lipid agar in the form of a large 'Z' and incubated for 48h at 28°C. Approximately 20 surface sterile IJ nematodes were added and the plates were further incubated at 25°C. At the time of IJ formation after growth and development (approximately 3 weeks) a sample of bacteria growing on the plate was taken from three separate areas, pooled together, and serially diluted in LB. These cells were plated out onto selective media to differentiate between the two strains to determine the ratio of bacteria present. The IJ nematodes were collected and crushed as described and the colonies recovered were plated out onto selective media to differentiate between the two strains.

2.7.2 Retention of *P. luminescens* by *Heterorhabditis*

Nematodes carrying *P. luminescens* were surface sterilised, by washes in 0.4% (w/v) hyamine and 3 washes of sterile PBS. The density of IJs in PBS was calculated and 1000 nematodes were crushed between two glass slides. The suspension on the slide was kept moist with drops of sterile LB, resuspended in a final total of 300µl LB and 100µl of an appropriate dilution were plated out. Alternatively nematodes were crushed using approximately 200µl glass beads in a total of 800µl LB/nematode containing PBS in a sterile eppendorf. The eppendorf was then vortexed for 15mins and 100µl plated out.

2.8 Generating Spontaneous Rifampicin Resistance

P. luminescens TT01 was grown overnight as described and 10mls were spun down and resuspended in 100µl LB, this was spread onto an LB plate containing rifampicin using a sterile spreader. The plate was incubated at 28°C for 48h, colonies of spontaneous rifampicin resistant mutants were picked and streaked onto LB plates containing rifampicin to check the resistance was stable.

2.9 Conjugation

Rifampicin resistant *P. luminescens* TT01 was grown overnight as described and 1ml was inoculated into 50ml LB in a 250ml conical flask. This was incubated to an OD_{600} of 0.5 and 4ml were spun down and washed twice in 200µl LB. *E. coli* S17-1 pLOF-Kn (Herrero *et al.*, 1990) was grown overnight as described and 1ml was inoculated into 50ml LB in a 250ml conical flask. This was incubated to an OD_{600} of 0.5, 1ml was spun down and washed twice in LB and finally suspended in 100µl. These washed suspensions of *P. luminescens* and *E. coli* were mixed together in a sterile eppendorf and the resulting 300µl were pipetted onto an LB agar plate containing no antibiotics and left undisturbed at 25°C overnight. The exconjugants were recovered by pipetting 1ml of LB onto the agar plate and carefully resuspending the bacteria with a sterile spreader. The resuspended cells were drawn off with a pipette and transferred to a sterile 14ml tube. This was repeated three times and 100µl of the final cell suspension (Total of 3ml) were then plated out onto LB plates containing rifampicin and kanamycin. Resulting mutants were checked for ampicilin resistance to confirm that the delivery plasmid was not present.

The plasmid pLOF-Kn has an R6K replication origin and is only able to replicate in host strains that produce π protein encoded by the *pir* gene. Once the plasmid has been conjugally transferred to the recipient strain (without the *pir* gene) it cannot replicate and be passed on to daughter cells. The transposable kanamycin resistance cassette is flanked by two inverted repeats which are recognised by the transposase and the kanamycin resistance cassette in between is cut out of the plasmid and randomly inserted into the hosts genome.

2.10 Motility Screen

P. luminescens mutants were transferred using sterile toothpicks to swim agar plates and grown overnight at 28°C. Mutants that appeared defective in this primary screen were then grown overnight in liquid culture as described and resuspended to OD_{600} of 1.0, 3µl of this suspension were then pipetted onto swim agar and left to incubate overnight.

2.11 Electron Microscopy

Bacterial flagella were visualised using negative staining. Bacteria were adsorbed to a carbon coated TEM grid and negatively stained with 4% aqueous uranyl acetate. Grids were examined in a JEOL 1200EXII transmission electron microscope operating at 80KV.

2.12 Genomic DNA Extraction

Genomic DNA from the bacterial strain of interest was extracted using methods adapted from standard procedures (Sambrook *et al.,* 1989). The bacterial strain of interest was grown in suspension overnight in 3mls LB, the culture spun in a centrifuge and the supernatant was discarded. The pellet was resuspended in 567µl TE buffer by repeated pipetting, and transferred to an eppendorf, 30µl of 10% SDS, 3µl of 20mg/ml proteinase K and 3µl RNase were then added. This was mixed thoroughly and incubated for 1h in a water bath at 37°C, 100µl of 5M NaCl and 80µl of pre-heated CTAB/NaCl (65°C) were added and the eppendorf was mixed thoroughly. This was left to incubate at 65°C for 10 minutes with occasional inverting. An approximately equal volume (0.7-0.8ml) of chloroform/isoamylalcohol was added and the eppendorf was thoroughly mixed and spun in a microcentrifuge at 10,000rpm for 5 minutes. The aqueous, viscous supernatant was transferred to a fresh microcentrifuge tube and an equal volume of phenol/chloroform/isoamyl alcohol was added. The eppendorf was thoroughly mixed and spun in a microcentrifuge at 10,000rpm for 5 minutes. The supernatant was transferred to a fresh tube and 0.6 volume isopropanol was added to precipitate the nucleic acids and the tube was left at -80°C for at least 30 minutes. The eppendorf was then spun in a microcentrifuge at room temperature and 13,200rpm for 10 minutes to pellet the DNA, the supernatant was discarded

and the pellet was washed in ice cold 70% ethanol. This was then spun again under the same conditions and the supernatant discarded, the pellet was allowed to air dry and 100µl sterile nuclease free water were added to resuspend the DNA.

2.13 Electrophoresis of DNA

DNA was electrophoresed on a 1% agarose (BDH) TAE (4.84g Tris, 1.14ml acetic acid, 2ml EDTA pH8.0 per litre) gel containing 100µg/ml ethidium bromide in a 1x TAE buffer. Promega ladder and loading dye were used according to manufactures instructions and the gels were run at 100V and the DNA visualised in a UV transilluminator.

2.14 DNA Gel Extraction

The desired band was cut out of an agarose gel and placed in an eppendorf, the gel slice was weighed and 6M NaI was added (3ml/g). The eppendorf was incubated at 55°C for 10 minutes and 20µl silica beads suspended in 6M NaI were added. The eppendorf was incubated at room temperature with constant agitation for 15mins. The silica beads were pelleted in a microcentrifuge at room temperature at 13,200 rpm for 30 seconds and then the supernatant was discarded and the pellets resuspended in ice-cold wash buffer (10mM Tris-HCl, pH 7.5, 50mM NaCl, 2.5mM EDTA, 50% ethanol), this wash process was repeated twice more. The silica pellets were resuspended in 20µl sdH_2O to elute the bound DNA and incubated at room temperature for 5mins before being pelleted once more. The supernatant was carefully removed and stored at -20°C until needed.

2.15 Electroporation of *E. coli*

Electrocompetent *E.coli* cells were prepared by inoculating 100ml of LB with 200µl overnight culture and growing to mid-exponential phase (OD_{600} 0.5-0.7). The cells were then harvested by centrifugation at 4,000rpm for 10 minutes at 4°C, the supernatant was discarded and the pellet resuspended in 100ml ice-cold sdH_2O. The cells were harvested as above and resuspended in 50ml ice cold sdH_2O then 1.6ml ice cold sterile 20% (v/v) glycerol and finally in 160µl ice cold sterile 20% (v/v) glycerol and frozen at -80°C until needed.

2.15.1 Electroporation of *Photorhabdus*

Electrocompetent *Photorhabdus* cells were prepared by inoculating 100ml of LB with 200µl overnight culture and growing the culture to early exponential phase [OD_{600} 0.2-0.3] before placing on ice for 90 minutes. The cells were harvested by centrifugation at 4,000rpm for 10 minutes at 4°C and the pellet was resuspended in 100ml ice-cold 5% sucrose, 1mM HEPES. The cells were harvested as above and resuspended in 50ml then 1.6ml and finally in 160µl ice cold 5% sucrose, 1mM HEPES. For *Photorhabdus luminescens* TT01 BMM305, the pellets were resuspended in ice-cold 500mM HEPES in the same quantities. The cells were used straight away and not stored.

2.16 Cloning of interrupted DNA

Genomic DNA was digested overnight with *Eco*RV and cleaned using phenol extraction and ethanol precipitation. The plasmid pBR322 was purified and cut with *Eco*RV, the ends were dephosphorylated using CIAP (Promega) and following manufactures instructions. The cut genomic and plasmid DNA were ligated together overnight, the plasmids were electroporated into *E. coli* JM109 and plated out onto selective media. Kanamycin and ampicilin resistant colonies were selected and grown in liquid overnight and the plasmids recovered. Plasmid DNA was then cut with *Eco*RV to check insert size. PCR was performed using the primers pBR322F (5'-GTCATCCTCGGCACCGTCACCCTGG-3') and KanATGR (5'-AGACGTTTCCCGTTGAATATGGCTCAT-3'). The appropriate sized band was cut out of the gel and purified using the above method and sent for sequencing using the primer Tn5R4 (5'-CCGTTGCGCTGCCCGGATTACAGCC-3'). Primers pBR332F and pBR322R (5'-CCAAAGCGGTCGGACAGTGCTCCGAG-3') were also used for sequencing the area surrounding the transposon.

2.17 Complementation

The *pbgE1* gene was amplified from *P. luminescens* TT01 chromosomal DNA with the primers pmrKF (5'-TCGAGCCATGGTTGAATAACCGGGCGTGTAAGG-3') and pmrKR (5'-ACGGCTCTAGATCATGGTTGCTTCTCATAAACC). The ~1660bp PCR

product was digested with *Nco1* and *Xba1* sites present in primers and cloned into pTRC99a to make plasmid pBMM500. The plasmid was then electroporated into *P. luminescens* TT01 Rif and *P. luminescens* TT01 BMM305. The genes *pbgE2, pbgE3* and *phoP* were amplified using the following primers pmrLF (5'-TCGCGCCATGGTAAGTTTCTCTTTATTGTTGC-3'), pmrLR (5'-ACGGCTCTAGATCATTCAAGCTCATCAGC-3'), pmrMF (5'-TCGCGCCATGGAAGGTTATATTTGGGG-3'), pmrMR (5'-ACGGCTCTAGATCATTCTGGACGGCTAATC-3'), phoPF (5'-TCGAGCCATGGGGATATTGATCGTTGAAGACAAC-3'), and phoPR (5'-ATAGCTCTAGATTACACATCGAAGCGATAGCC-3') to generate the plasmids pBMM501, pBMM502 and pBMM505 respectively. Combinations *pbgE1E2E3* and *pbgE2E3* were amplified using the appropriate combinations of primers to create the plasmids pBMM503 and pBMM504 respectively.

2.18 Detection of Kanamycin Cassette

2.18.1 Southern Blotting

*Eco*RI cut genomic DNA was run on an agarose DNA gel, the gel was denatured in 300ml denaturing solution (1.5M NaCl, 0.5M NaOH) for 30 mins at room temperature with gentile agitation, the solution was poured off and fresh denaturing solution was added and incubated for a further 15 mins. The gel was rinsed with ddH_2O before being washed in 300ml neutralisation solution (1M TRIS pH 7.4, 1.5M NaCl) at room temperature with gentile agitation for 30 minutes. The solution was poured off and fresh neutralisation solution was added and incubated for a further 15 mins. The DNA was transferred to a nitrocellulose membrane (Hybond-N^+, Amersham Pharmacia Biotech) using a Southern blotting technique based on capillary transfer using 20X SSC (175.3g NaCl, 88.2g NaCi/l pH 7.0).

2.18.2 DIG labelling of DNA Probe and Detection

DIG labelling and detection was performed using the DIG-High Prime DNA Labelling and Detection Starter Kit II (Roche). Template DNA was denatured by boiling in a water bath for 10 minutes before quickly chilling in an ice/water bath. Premixed DIG-High Prime was added to the denatured DNA and mixed

again before being briefly centrifuged. This was then incubated overnight at 37°C. The reaction was stopped by adding 0.2M EDTA (pH8.0) and heating to 65°C for 10 minutes. An appropriate volume of DIG Easy Hyb (10ml/100cm^2 filter) was pre-heated in a hybridisation bottle to 40°C, the membrane was added and prehybridised for 30 minutes with gentle agitation. The DIG-labelled DNA probe (approximately 25ng/ml DIG Easy Hyb) was denatured by boiling for 5 minutes and then rapidly cooled in ice/water. This was then added to pre-heated Dig Easy Hyb (3.5ml/100cm^2). The prehybridisation solution was poured off and the probe/hybridisation mixture was added to the membrane. This was then incubated at 40°C overnight with constant agitation.

The membrane was washed twice for 5 minutes in ample 2x SSC, 0.1% SDS at 20°C under constant agitation, then washed twice for 15 minutes in 0.5x SSC, 0.1% SDS prewarmed to 65°C under constant agitation. The membrane (100cm^2) was then rinsed briefly (1-5min) in washing buffer (0.1M maleic acid, 0.15M NaCl; pH 7.5 (20°C); 0.3% (v/v) Tween 20), then incubated for 30 minutes in 100ml blocking solution this was 10x blocking solution (provided) diluted 1:10 in maleic acid buffer (0.1M Maleic acid, 0.15M NaCl; adjust with NaOH (solid) to pH 7.5 (20°C). The membrane was incubated for 30 minutes in antibody solution (anti-digoxigenin-AP was centrifuged for 5 minutes at 10,000rpm, 2µl were added to 20ml blocking solution) the membrane was then washed twice for 15 minutes in 100ml washing buffer, it was then equilibrated for 2-5mins in 20ml detection buffer (0.1M Tris-HCl, 0.1M NaCl, pH 9.5 (20°C). The membrane was placed DNA side facing up on cling film, 1ml CSPD™ was added evenly to the membrane. The membrane was immediately covered with a second sheet of cling film and incubated for 5 minutes at room temperature. The excess liquid was squeezed out and the edges of the cling film sealed, the damp membrane was further incubated at 37°C for 10 minutes. Finally the membrane was exposed to X-ray film for an appropriate amount of time.

2.19 Isolation and SDS-PAGE Analysis of Lipopolysaccharide

LPS was isolated from *P. luminescens* TT01 by the following method adapted from Dunphy and Webster (1991). Briefly, 25ml of overnight culture were

pelletted at 4000rpm for 15 minutes at room temperature and the supernatant discarded. Then 5ml of 0.5M EDTA (pH8.0) were added and the pellet resuspended, this suspension was then agitated on a Bibby Stuart Science Blood Tube Rotator SB1 for 5h at room temperature. The suspension was then pelletted at 13,000rpm for 3 minutes and the supernatant retained and removed to a fresh eppendorf. This was then centrifuged at 13,000 for 20 minutes at room temperature. The supernatant was discarded and the pellet resuspended in 10µl water, 1µl proteinase K was added and the sample incubated at 37°C for 1h.

LPS was also isolated from *P. luminescens* using a second method. The strain of interest was grown overnight and 1.5ml were pelletted in a microcentrifuge. This pellet was resuspended in 0.5ml LB and pelletted again, this wash was repeated with 0.5ml PBS. To this PBS cell suspension 0.5ml phenol was added (room temp). The eppendorf was inverted to mix the contents and then centrifuged for 10 mins at room temperature at 10,000rpm. The aqueous layer was removed to a fresh eppendorf and 1ml of ethanol was added. The contents were then centrifuged for 10 minutes at 13,200rpm and supernatant discarded, the pellet was washed in 70% ethanol and spun to pellet (10 mins 13,200rpm) once more. The supernatant was discarded and the pellet washed in 99% ethanol. The contents were pelletted again and the supernatant discarded. Finally the pellet was resuspended in 10µl sdH_2O, 1µl proteinase K was added and the sample incubated at 37°C for 1h.

The sample had 10µl 2x loading dye (125mM tris pH6.8, 10% β-mercaptoethanol 4% SDS, 20% glycerol, 0.05% bromophenol blue) added and was boiled at 100°C for 10 minutes then placed immediately on ice. The samples collected were run on a 12.5% resolving gel (5mls = 2.5ml Buffer A (0.75M tris pH 8.0, 0.2% SDS), 1.56ml 40% acrylamide, 0.25ml 2% bisacrylamide, 0.69ml dH_2O, 25µl ammonium persulphate (APS), 7.0µl TEMED) and 5% Stacking gel (2mls = 1ml Buffer B (0.25M tris pH 6.8, 0.2% SDS), 0.25ml 40% acrylamide, 0.14ml 2% bisacrylamide, 0.6ml dH_2O, 12.5µl APS, 3.5µl TEMED) The running buffer was 250mM TRIS, 1.92mM glycine, 1.0% SDS. Gels were run at a

constant 20mA until the dye front was 1cm above the end of the glass plate and the LPS was visualised by silver staining.

2.19.1 Silver Staining

The gel was placed in 50% methanol overnight and then washed in 5% methanol for 5 mins. The gel was then placed in fixer (7:2:1 dH_2O:acetic acid:methanol) for 2x 30 mins. 0.7% (w/v) periodic acid (Sigma) was added to 100ml fixer and the gel washed for a further 5 mins. The gel was washed with dH_2O 3 times and placed in 32.5μM DTT, for 20 mins, then placed in 0.1% silver nitrate for a further 20 mins. The gel was then washed with dH_2O and rinsed 3 times with developer (3% sodium carbonate, 50μl 37.5% formaldehyde per 100ml) before longer exposure to developer. The reaction was stopped with the addition of 5ml 2.3M citric acid per 100mls developer.

CHAPTER 3

3.0 Characterisation of Motility Mutants.

3.1 Introduction.

A common form of bacterial motility is swimming, which is the movement of a bacterial cell through liquid or semisolid media by the organised rotation of flagella. Swimming is often associated with chemotaxis. The production, structure and function of flagella are well characterised and swimming motility has been implicated in both symbiosis and pathogenicity of bacteria towards their hosts (Macnab, 2003; Blair, 1995; Otterman and Miller, 1997). A second widespread form of movement is swarming, which is the movement of cells over a solid media. This is also mediated by flagella; however swarming is a more complex phenomenon and involves the up-regulation of a number of genes as well as cell-to-cell coordination, communication and cell differentiation (Fraser and Hughes, 1999). A third type of motility is flagella independent and called gliding, and a subdivision of gliding includes a form of motility called social gliding or twitching (Wall and Kaiser, 1999). Twitching is mediated by type IV pili and is the movement of cells involving a surface structure, typically between either two solid surfaces or a solid and a semisolid surface such as the plastic of a petri dish and the agar. The ability of a bacterial cell to move can confer an advantage to the cell or have a crucial function in its host interactions. In this chapter the ability of *Photorhabdus luminescens* TT01 to be motile was investigated and random transposon mutagenesis was used to create non-motile (non-swimming) mutants, which were then characterised.

3.2. Results.

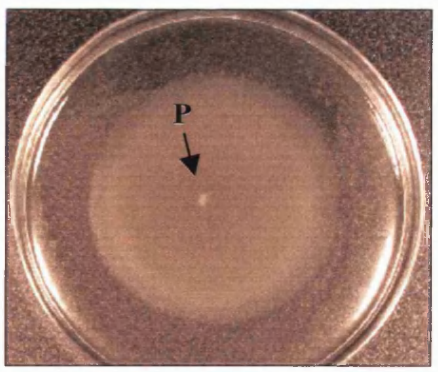
3.2.1 Motility.

P. luminescens was grown up over night in liquid culture, 3µl were pipetted onto 0.3% LB agar and the plate was incubated at 28°C for 24h. A colony formed at the site of initial inoculation and an opaque halo was visible surrounding the colony, after further incubation the halo was noticeably bigger (Fig. 3.0 A).

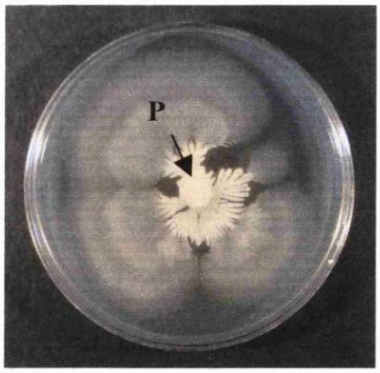
Microscopic examination of a section of the agar clearly showed swimming and tumbling cells. Therefore *P. luminescens* TT01 is able to swim through semisolid media. Different concentrations of LB agar were made to try and induce swarming and these were inoculated with *P. luminescens* TT01 and incubated at 28°C. Swarming was observed when TT01 was inoculated onto LB agar containing 0.9% agar. After 24h the colony appeared to be very sticky and shiny and larger than usual, upon further incubation, protrusions started to appear from the colony edges and could be observed over the course of a few hours to grow larger (Fig. 3.0 B). Macroscopically, this growth appeared to undergo a consolidation phase with the central area becoming more opaque indicating growth, before more protrusions appeared and spread outwards. This cycle continued until the whole plate was confluent with TT01 growth, although rings of growth could still be observed. Therefore under the appropriate conditions *P. luminescens* TT01 can swarm; however swarming was difficult to reproduce reliably. Twitching is the movement of bacterial cells, using type IV pili, between a solid surface and either another solid surface or semisolid surface (Skerker and Berg, 2001). A sterile toothpick was used to inoculate TT01 to the bottom of an agar plate and the plate was incubated at 28°C for 24h. A colony formed on the surface of the agar plate where the toothpick pierced the agar. At the bottom of the agar dish where the toothpick touched the plastic dish a small transparent film of growth appeared as an irregular halo (Fig. 3.0 C); therefore *P. luminescens* TT01 is able to twitch. Upon further incubation the halo did not get significantly bigger but did become more opaque indicating an increase in the number of cells present.

3.2.2 Generation of Non-Motile Mutants

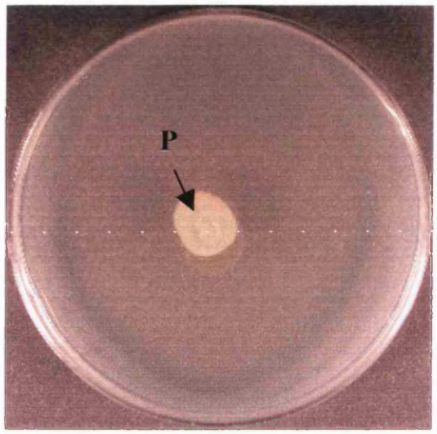
In order to obtain strains of *P. luminescens* TT01 unable to swim or twitch random transposon mutagenesis was performed. Rifampicin resistant *P. luminescens* TT01 was conjugated with *E. coli* S-17 carrying an ampicillin resistant suicide plasmid (pLOF-Kn) with a transposable kanamycin resistance cassette. Exconjugants were obtained as described in material and methods and kept for further study.



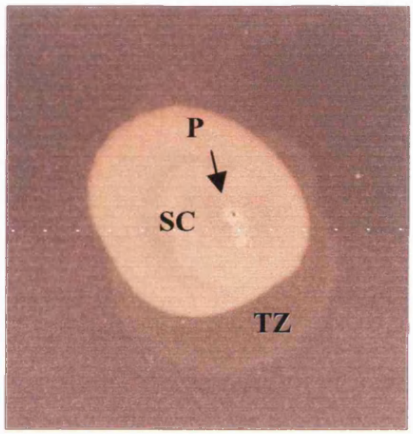
A)



B)



C.i.)



C.ii.)

P = Point of inoculation. SC = surface colony. TZ = Twitch zone.

Figure 3.0. Motility of *Photorhabdus luminescens* TT01. An overnight culture of *P. luminescens* TT01 was prepared as described in materials and methods. A) 3µl of culture were inoculated onto a swim (0.3% w/v) LB agar plate. B) 3µl was inoculated onto a swarm agar plate (0.9% w/v) LB agar plate C.i. and C.ii.) A sterile pipette tip containing 1µl overnight culture was used to pierce through to the bottom of a 1.5% LB agar plate. Plates were incubated for 48h at 28°C.

3.2.3 Isolation of Twitching Mutants

Twitching is a specialised type of motility between solid and semisolid media and involves type IV pili. A gene with homology to *pilA*, a type IV pili biogenesis gene, was shown to affect the symbiotic competitive colonisation of

Euprymna scolopes by *Vibrio fischeri* (Stabb and Ruby, 2003). Therefore in order to assess whether twitching was important in *Photorhabdus* host interactions random transposon mutants were screened for the ability to twitch (Fig. 3.0). Over 4000 mutants were screened for twitching defects; however a twitching minus mutant could not be isolated. This could be due to two reasons, redundancy or hot-spotting, *Photorhabdus* is known to have duplicate copies of a number of genes on the chromosome. Specifically TT01 has been reported to have approximately 80 genes involved in pili biogenesis, including two very similar *pil* clusters containing 10 genes each that have homology to type IV pili genes (Duchaud *et al.*, 2003). This suggests that TT01 may have duplicate copies of the type IV pili genes making a knockout using the above system difficult. Furthermore, although this method of making knockouts is described as random transposon mutagenesis, certain areas of the chromosome are more likely to receive the transposon than others. This is due to the secondary structure of DNA caused by chromosome folding and is known as transposon hot spotting. The area of the chromosome containing the pili genes may also be unfavourable leading to a vastly reduced chance of insertion.

3.2.4 Isolation and analysis of Swimming Mutants

E. coli and *Salmonella* both have approximately 50 genes that are involved in motility and chemotaxis and they can be divided into 17 operons (Macnab, 1996). The operons themselves can be divided into 3 classes. The first class is the early-transcribed genes, which includes just one operon, *flhDC*, which is necessary for the transcription of class II and III. Class II genes include many of the structural components for the basal body and hook and also *fliA*, which encodes a sigma factor for the expression of late or class III genes. The class III genes include those that encode the flagella filament structural proteins and the associated chemotaxis machinery. Mutants in motility may lay in any of these classes with severe defects likely to be structural mutants or mutants in class I.

In silico analysis of the recently published TT01 genome has revealed that TT01 is predicted to share 48 genes (See chapter 1, table 1.0) involved in flagella biosynthesis and function with other Gram-negative motile organisms, such as

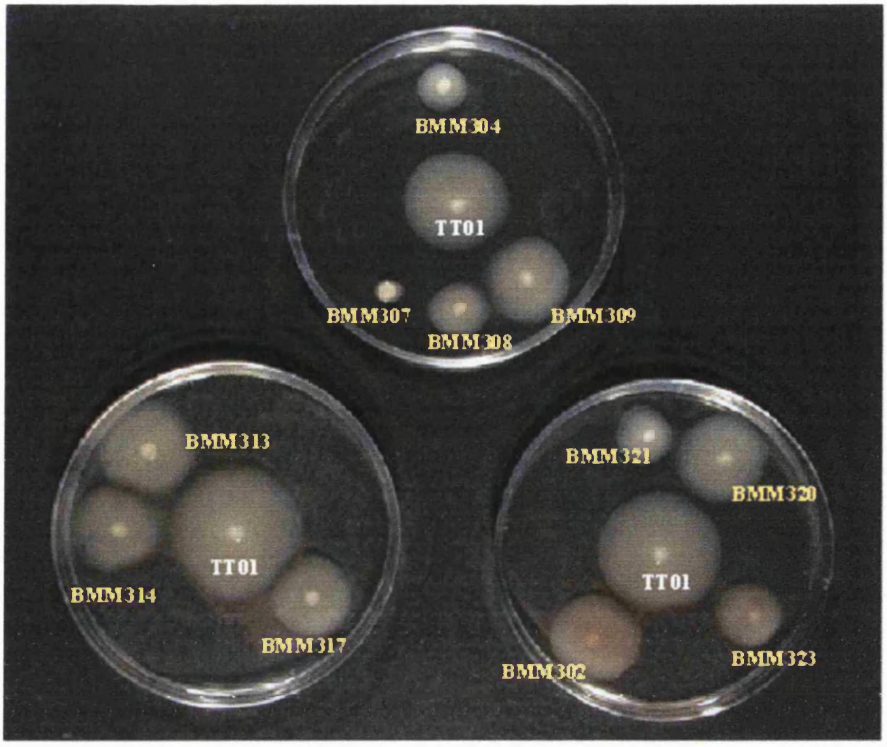


Figure 3.1. Transposon mutants on swim agar. Each mutant was grown overnight as described in Materials and Methods and 3µl pipetted onto swim agar. The plates were incubated for 48h at 28°C (the motility of BMM316 and BMM305 are included in Fig. 4.0 and 5.2 A respectively).

E. coli and *Salmonella*, and these genes do not appear to be duplicated on the TT01 chromosome. Approximately 3000 random transposon mutants were screened for the ability to swim in semi solid agar. In this way, 13 independent mutants were isolated as being defective in motility to varying degrees when compared to TT01 (Fig. 3.1). BMM316 and BMM305 were studied further and are included in separate figures in chapter 4 (Fig. 4.0) and chapter 5 (Fig. 5.2 A) respectively. Two mutants, BMM307 and BMM316, were severely impaired in motility after 48h incubation indicating that the transposon may have inserted in genes critical for flagella production (Table 3.0). The mutants were also screened for the ability to swarm and twitch and all the mutants, except BMM307 and BMM316, retained the ability to swarm on the appropriate agar (Table 3.0) and all mutants could twitch.

Strain	Swim	Swarm	Twitch
TT01	++++	+	+
BMM302	++	+	+
BMM304	++	+	+
BMM305	++	+	+
BMM307	-	-	+
BMM308	++	+	+
BMM309	++	+	+
BMM313	+++	+	+
BMM314	+++	+	+
BMM316	-	-	+
BMM317	+++	+	+
BMM320	+++	+	+
BMM321	++	+	+
BMM323	+++	+	+

Key. - = negative result. + = positive result. S+ = slow

Table 3.0. Table of motility of mutants. Mutants were assessed on their ability to swim, swarm and twitch compared to TT01. BMM316 remained non motile, BMM307 was severely attenuated and the other mutants were motile to varying degrees. BMM316 and BMM307 could not swarm.

Genomic DNA was obtained from each of the mutants and digested with *Eco*R1. Southern Blot analysis using the kanamycin resistance gene carried by the insertion revealed each mutant to have a single insertion (Fig. 3.14). The site of insertion for each mutant was identified by subcloning the chromosomal Eco*RV* fragment containing the kanamycin resistance gene into pBR322 (Table 3.1). The mutants that were obtained from this screen could be divided into 3 main groups, those with inserts in genes encoding proteins with homology to proteins directly related to motility, those with homology to membrane proteins and those with homology to metabolic and catabolic enzymes. A fourth group describes genes encoding proteins with no obvious direct or indirect role in motility.

Mutant	Gene (*plu*)	Insert location	Function	Homology/Organism	Further information
Group I. Flagella genes					
BMM307	*flhD*	125bp US	flagella transcriptional activator	88%ID 97%SIM flagella transcriptional activator, FlhD. *Xenorhabdus nematophila*	Figure 3.5. Table 3.5
BMM309	*fliT*	180bp DS	Flagellar protein (repressor of class 3a and 3b operons)	50%ID 69%SIM FliT *Yersinia pestis* CO92	Figure 3.7. Table 3.7.
BMM316	*flgG*	705bp	Flagellar basal-body rod protein	77%ID 83%SIM Flagellar basal-body rod protein *Salmonella enterica* serovar Typhi Ty2	Figure 3.10. Table 3.10.
Group II. Membrane genes.					
BMM305	*pbgE1*	1281bp	LPS biosynthesis	57%ID 72%SIM dolichyl-phosphate-mannose-protein *Yersinia pestis* CO92	Figure 3.3. Table 3.3.

BMM308	*sdaC*	551bp US	Serine transporter	73%ID 81%SIM Serine transporter, SdaC. *Yersinia pestis* CO92	Figure 3.6. Table 3.6.
BMM320	*plu1627*	18bp DS		25%ID 40%SIM Putative membrane protein *Bacillus anthracis*	Figure 3.11. Table 3.11.

Group III. Metabolic genes

BMM302	*ngrA*	24bp	4'-phosphopantetheinyl transferase	90%ID 95%SIM NgrA *Photorhabdus luminescens* subsp. *akhurstii* W14	Figure 3.2. Table 3.2.
BMM314	*plu0756*	579bp		31%ID 47%SIM Non-ribosomal peptide synthetase modules and related proteins *Burkholderia fungorum*	Figure 3.9. Table 3.9.
BMM317	*plu0753*	33bp		29%ID 48%SIM 3-oxoacyl-[acylcarrier-protein] synthase III *Burkholderia fungorum*	Figure 3.9. Table 3.9.
BMM321	*plu0643*	44bp		55%ID 69%SIM	Figure 3.12.

				serine protease, subtilase family *Pseudomonas putida* KT2440	Table 3.12.
BMM323	*plu2234*	148bp		51%ID 71%SIM Histidine ammonia-lyase (Histidase) *Dictyostelium discoideum*	Figure 3.13. Table 3.13.

Group IV. Genes with unknown putative roles in motility.

BMM304	*plu4230*	779bp		69%ID 81% SIM photopexin A *Photorhabdus luminescens* subsp. *laumondii* TTO1	Figure 3.4. Table 3.4.
BMM313	*plu4460*	365bp	Putative intergrase	67%ID 77%SIM intergrase *Salmonella enterica* serovar Typhi Ty2	Figure 3.8. Table 3.8.

Table 3.1. Table of mutants grouped into four main groups. Table also includes location of insertion in gene (US = up stream, DS = down stream), function of respective gene and homology (ID = Identical, SIM = similar) at the predicted protein level to protein products in other bacteria. The last column references further information.

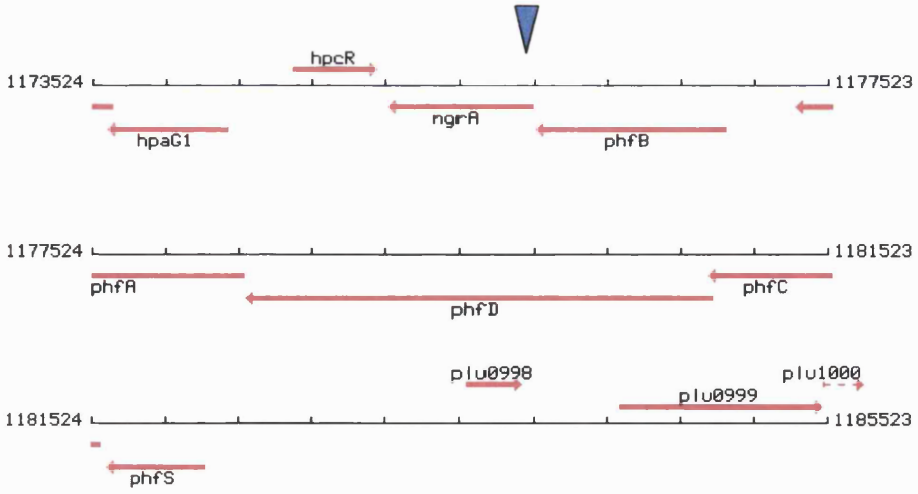


Figure 3.2. BMM302 insertion site and surrounding genes. Blue arrow represents point of insertion. Red arrows represent coding sequences.

Gene	Gene length	Coordinates / Orientation	Orient-ation	Description
hpaG1	633	1173611..1174243	-	Unknown.
hpcR	447	1174630..1175076	+	homoprotocatechuate degradation operon regulator
ngrA	**744**	**1175153..1175896**	-	**4'-phosphopantetheinyl tranferase**
phfB	1011	1175942..1176952	-	Putative fimbrial adhesin
phfA	981	1177360..1178340	-	Putative fimbrial adhesin
phfD	2514	1178368..1180881	-	Putative export and assembly of fimbrial adhesin
phfC	669	1180887..1181555	-	Putative fimbrial chaperone
phfS	510	1181612..1182121	-	Probable fimbrial adhesin
plu0998	294	1183567..1183860	+	3-oxoacyl-(acyl carrier protein) reductase
plu0999	1095	1184406..1185500	+	Unknown

Table 3.2. Table of genes surrounding insertion site in BMM302. Table includes gene name, length, coordinates on the *P. luminescens* chromosome, orientation and brief description. Bold type indicates gene interrupted in BMM302.

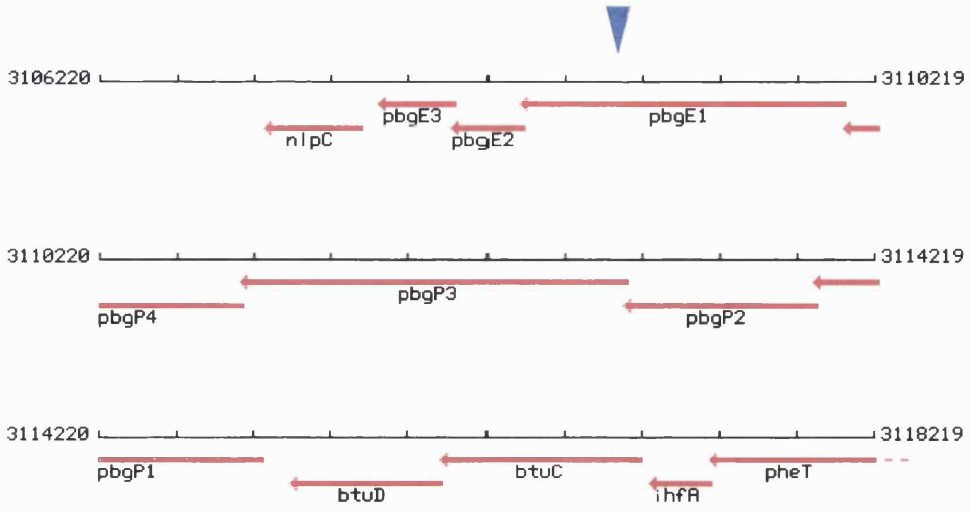


Figure 3.3. BMM305 insertion site and surrounding genes. Blue arrow represents point of insertion. Red arrows represent coding sequences.

Gene	Gene length	Coordinates	Orient-ation	Description
nlpC	486	3107082..3107567	-	Probable lipoprotein NlpC precursor
pbgE3	381	3107673..3108053	-	PbgE3 protein
pbgE2	342	3108050..3108391	-	PbgE2 protein
pbgE1	**1662**	**3108388..3110049**	-	**PbgE1 protein**
pbgP4	894	3110057..3110950	-	PbgP4 protein
pbgP3	1983	3110950..3112932	-	PbgP3 protein
pbgP2	978	3112933..3113910	-	PbgP2 protein
pbgP1	1146	3113911..3115056	-	PbgP1 protein
btuD	777	3115217..3115993	-	Vitamin B12 transport ATP-binding protein BtuD
btuC	1008	3115997..3117004	-	Vitamin B12 transport system permease protein BtuC
ihfA	297	3117070..3117366	-	Integration host factor α chain

Table 3.3. Table of genes surrounding insertion site in BMM305. Table includes gene name, length, coordinates on the *P. luminescens* chromosome, orientation and brief description. Bold type indicates gene interrupted in BMM305.

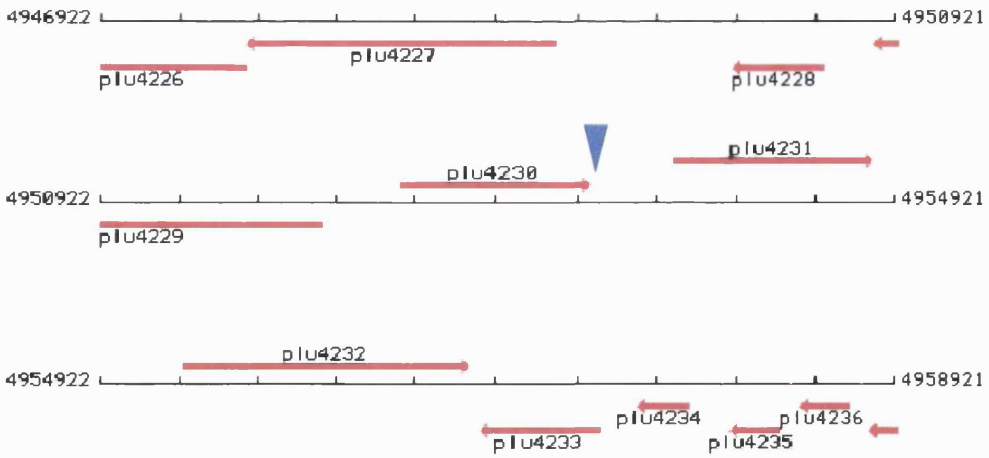


Figure 3.4. BMM304 insertion site and surrounding genes. Blue arrow represents point of insertion. Red arrows represent coding sequences.

Gene	Gene length	Coordinates	Orient-ation	Description
plu4227	1533	4947666..4949198	-	Hypothetical protein
plu4228	441	4950105..4950545	-	Hypothetical protein
plu4229	1212	4950825..4952036	-	Putative multidrug-resistance protein
plu4230	**939**	**4952453..4953391**	+	**Unknown, homology to photopexin A/B**
plu4231	1002	4953811..4954812	+	Homology to photopexin A
plu4232	1449	4955352..4956800	+	Probable monooxygenase
plu4233	561	4956862..4957422	-	Uncharacterized membrane protein
plu4234	237	4957634..4957870	-	Unknown
plu4235	225	4958100..4958324	-	Unknown
plu4236	225	4958454..4958678	-	Unknown

Table 3.4. Table of genes surrounding insertion site in BMM304. Table includes gene name, length, coordinates on the *P. luminescens* chromosome, orientation and brief description. Bold type indicates gene interrupted in BMM304.

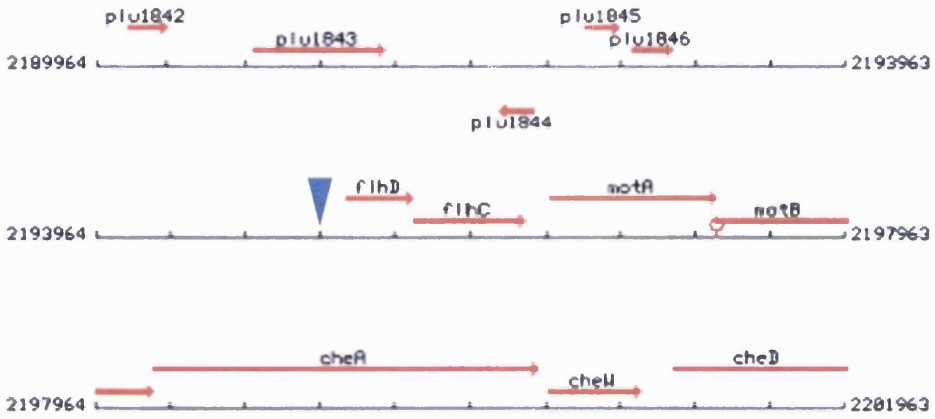


Figure 3.5. BMM307 insertion site and surrounding genes. Blue arrow represents point of insertion. Red arrows represent coding sequences.

Gene	Gene length	Coordinates	Orient-ation	Description
plu1842	213	2190134..2190346	+	Probable cold shock protein
plu1843	711	2190810..2191520	+	Mg(2+) transport protein C
plu1844	171	2192111..2192281	-	Unknown
plu1845	183	2192575..2192757	+	Unknown
plu1846	222	2192831..2193052	+	Unknown
flhD	**354**	**2195317..2195670**	+	**Flagellar transcriptional activator**
flhC	582	2195674..2196255	+	Flagellar transcriptional activator
motA	888	2196392..2197279	+	Chemotaxis protein
motB	1002	2197276..2198277	+	Chemotaxis protein
cheA	2052	2198280..2200331	+	Chemotaxis protein cheA
cheW	498	2200385..2200882	+	Chemotaxis protein
cheD	1695	2201059..2202753	+	Chemotaxis protein I (MCP-I)

Table 3.5. Table of genes surrounding insertion site in BMM307. Table includes gene name, length, coordinates on the *P. luminescens* chromosome, orientation and brief description. Bold type indicates gene interrupted in BMM307.

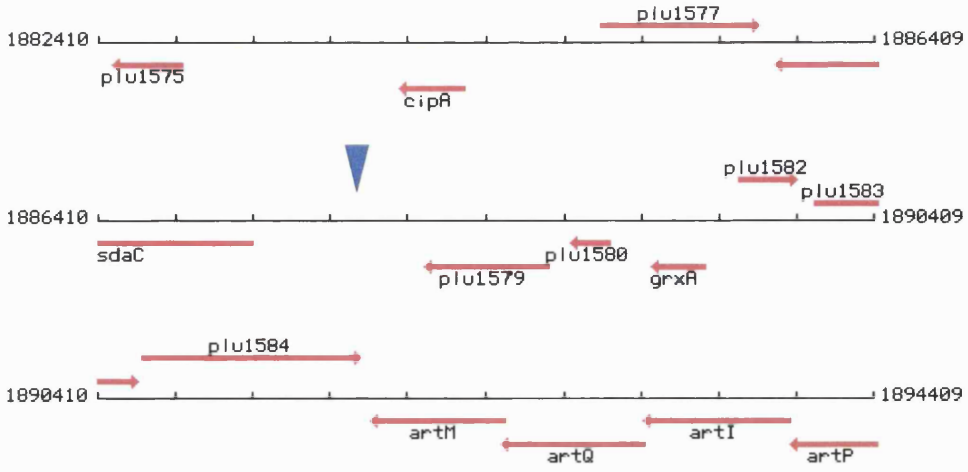


Figure 3.6. BMM308 insertion site and surrounding genes. Blue arrow represents point of insertion. Red arrows represent coding sequences.

Gene	Gene length	Coordinates	Orient-ation	Description
plu1575	339	1882485..1882823	-	Unknown
cipA	315	1883974..1884288	-	Crystalline inclusion protein CipA
plu1577	822	1885002..1885823	+	Unknown
sdaC	**1299**	**1885901..1887199**	-	**Serine transporter SdaC**
plu1579	**609**	**1888106..1888714**	-	**Probable permease**
plu1580	186	1888845..1889030	-	Unknown
grxA	261	1889263..1889523	-	Glutaredoxin 1 (Grx1)
plu1582	303	1889714..1890016	+	Putative membrane protein
plu1583	513	1890108..1890620	+	Hypothetical protein
plu1584	1134	1890645..1891778	+	Unknown.
artM	669	1891832..1892500	-	Arginine transport protein artM
artQ	717	1892500..1893216	-	Arginine transport protein artQ
artI	735	1893231..1893965	-	Arginine-binding protein
artP	729	1893985..1894713	-	Arginine transport protein ArtP

Table 3.6. Table of genes surrounding insertion site in BMM308. Table includes gene name, length, coordinates on the *P. luminescens* chromosome, orientation and brief description. Bold type indicates gene interrupted in BMM308.

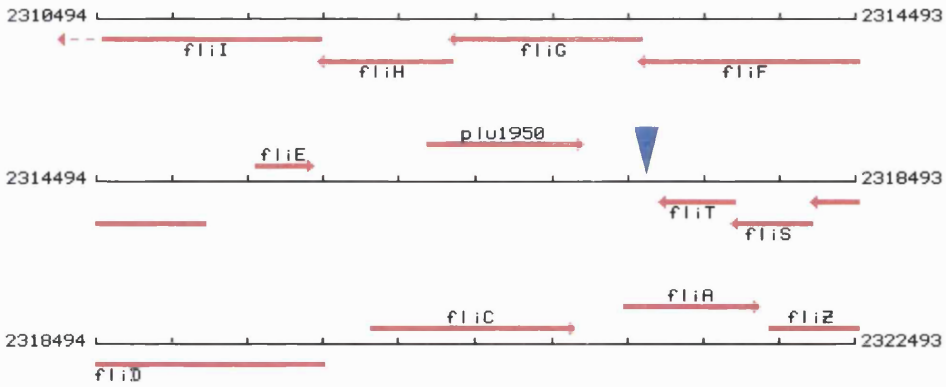


Figure 3.7. BMM309 insertion site and surrounding genes. Blue arrow represents point of insertion. Red arrows represent coding sequences.

Gene	Gene length	Coordinates	Orient-ation	Description
fliI	1365	2310303..2311667	-	flagellum-specific ATP synthase
fliH	705	2311667..2312371	-	Flagellar assembly protein FliH
fliG	993	2312364..2313356	-	Flagellar motor switch FliG
fliF	1704	2313353..2315056	-	flagellar basal-body M-ring protein
fliE	312	2315342..2315653	+	flagellar hook-basal body
plu1950	**813**	**2316251..2317063**	+	**Unknown**
fliT	**378**	**2317465..2317842**	-	**Flagellar protein FliT**
fliS	411	2317842..2318252	-	Flagellar protein FliS
fliD	1422	2318265..2319686	-	Flagellar hook-associated protein 2
fliC	1068	2319961..2321028	+	Flagellin
fliA	723	2321275..2321997	+	RNA polymerase sigma factor for flagellar operon (Sigma-28)
fliZ	513	2322050..2322562	+	FliZ protein

Table 3.7. Table of genes surrounding insertion site in BMM309. Table includes gene name, length, coordinates on the *P. luminescens* chromosome, orientation and brief description. Bold type indicates gene interrupted in BMM309.

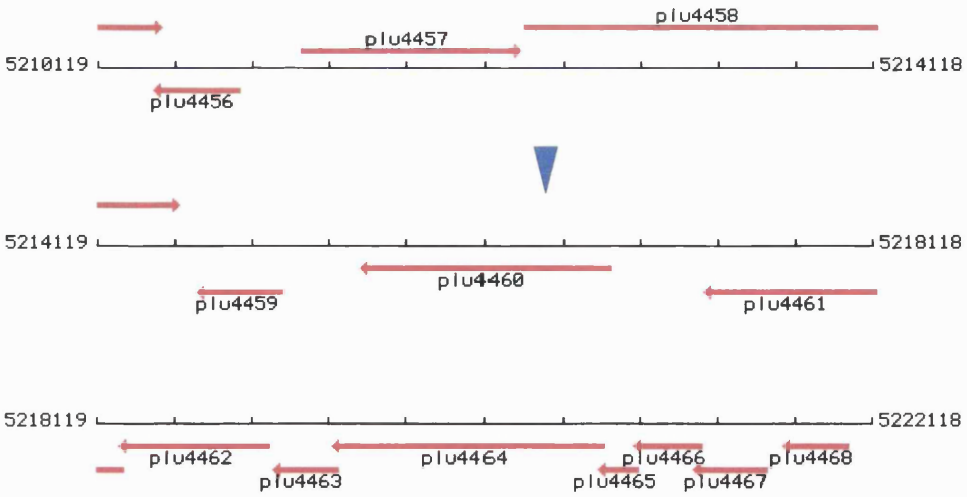


Figure 3.8. BMM313 insertion site and surrounding genes. Blue arrow represents point of insertion. Red arrows represent coding sequences.

Gene	Gene length	Coordinates	Orient-ation	Description
plu4456	423	5210412..5210834	-	Unknown
plu4457	1122	5211179..5212300	+	Probable ATPase
plu4458	2226	5212323..5214548	+	Probable ATPase
plu4459	432	5214642..5215073	-	Probable phage antitermination protein Q
plu4460	**1266**	**5215487..5216752**	-	**probable integrase**
plu4461	993	5217249..5218241	-	Unknown
plu4462	756	5218238..5218993	-	Unknown
plu4463	312	5219043..5219354	-	Unknown
plu4464	1377	5219344..5220720	-	Unknown
plu4465	183	5220713..5220895	-	Unknown
plu4466	324	5220898..5221221	-	Putative helix-turn-helix protein
plu4467	360	5221205..5221564	-	Putative cytoplasmic protein
plu4468	312	5221667..5221978	-	Unknown

Table 3.8. Table of genes surrounding insertion site in BMM313. Table includes gene name, length, coordinates on the *P. luminescens* chromosome, orientation and brief description. Bold type indicates gene interrupted in BMM313.

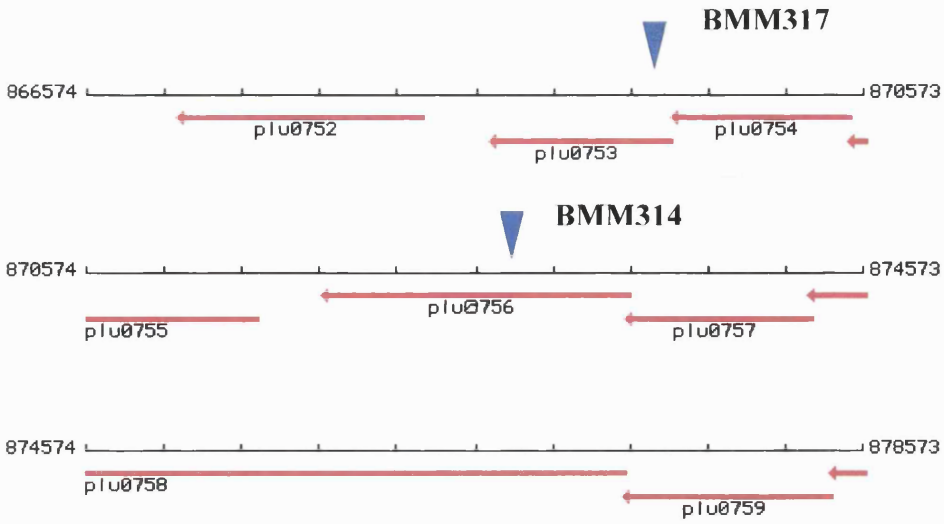


Figure 3.9. BMM314 and BMM317 insertion sites and surrounding genes. Blue arrow represents point of insertion. Red arrows represent coding sequences.

Gene	Gene length	Coordinates	Orient-ation	Description
plu0752	1260	867042..868301	-	Putative Orn/DAP/Arg decarboxylases family 2
plu0753	**939**	**868642..869580**	-	**Putative 3-oxoacyl-[acyl-carrier-protein] synthase III**
plu0754	915	869586..870500	-	Hypothetical protein
plu0755	960	870493..871452	-	Hypothetical protein
plu0756	**1566**	**871792..873357**	-	**Non-ribosomal peptide synthetase modules**
plu0757	948	873354..874301	-	Putative 3-oxoacyl-[acyl-carrier-protein] synthase III
plu0758	3042	874298..877339	-	Probable multidrug resistance protein
plu0759	1074	877336..878409	-	Probable efflux transporter, RND family, MFP subunit

Table 3.9. Table of genes surrounding insertion site in BMM314 and BMM317. Table includes gene name, length, coordinates on the *P. luminescens* chromosome, orientation and brief description. Bold type indicates gene interrupted in BMM314 and BMM317.

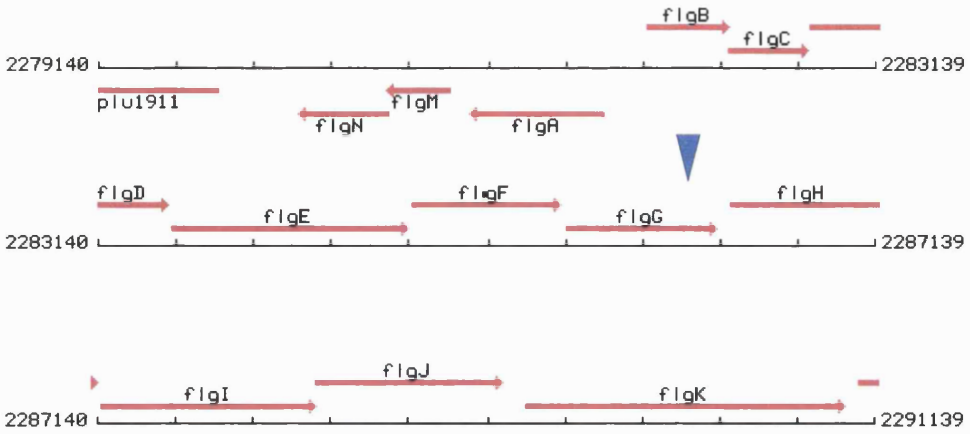


Figure 3.10. BMM316 insertion site and surrounding genes. Blue arrow represents point of insertion. Red arrows represent coding sequences.

Gene	Gene length	Coordinates	Orient-ation	Description
flgB	414	2281971..2282384	+	Flagellar basal-body rod protein FlgB (Putative proximal rod protein)
flgC	405	2282390..2282794	+	Flagellar basal-body rod protein FlgC (Putative proximal rod protein)
flgD	696	2282807..2283502	+	Basal-body rod modification protein FlgD
flgE	1215	2283530..2284744	+	Flagellar hook protein FlgE
flgF	756	2284763..2285518	+	Flagellar basal-body rod protein FlgF (Putative proximal rod protein)
flgG	**783**	**2285542..2286324**	+	**Flagellar basal-body rod protein FlgG (Distal rod protein)**
flgH	753	2286399..2287151	+	Flagellar L-ring protein precursor
flgI	1110	2287164..2288273	+	Flagellar P-ring protein precursor
flgJ	945	2288270..2289214	+	Peptidoglycan hydrolase FlgJ (Muramidase flgJ)
flgK	1650	2289341..2290990	+	Flagellar hook-associated protein

Table 3.10. Table of genes surrounding insertion site in BMM316. Table includes gene name, length, coordinates on the *P. luminescens* chromosome, orientation and brief description. Bold type indicates gene interrupted in BMM316.

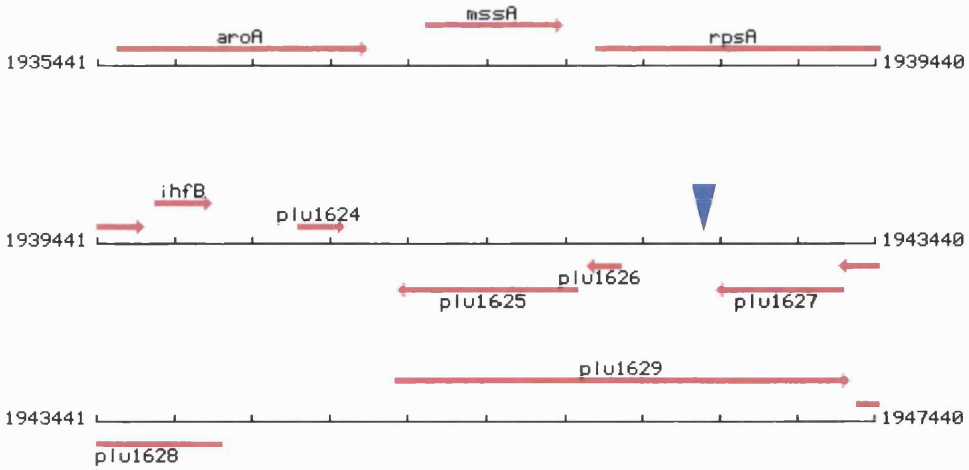


Figure 3.11. BMM320 insertion site and surrounding genes. Blue arrow represents point of insertion. Red arrows represent coding sequences.

Gene	Gene length	Coordinates	Orient-ation	Description
aroA	1287	1935549..1936835	+	3-phosphoshikimate 1-carboxyvinyltransferase (EPSP synthase) (EPSPS)
mssA	684	1937140..1937823	+	Cytidylate kinase (CK) (CMP kinase) (MssA protein) (P25)
rpsA	1674	1938005..1939678	+	30S ribosomal protein S1
ihfB	285	1939744..1940028	+	Integration host factor beta-subunit (IHF-beta)
plu1624	240	1940483..1940722	+	Unknown
plu1625	900	1940992..1941891	-	Unknown
plu1626	159	1941955..1942113	-	Unknown
plu1627	**636**	**1942624..1943259**	**-**	**Hypothetical protein**
plu1628	813	1943264..1944076	-	Conserved hypothetical protein
plu1629	2328	1944985..1947312	+	Putative membrane protein

Table 3.11. Table of genes surrounding insertion site in BMM320. Table includes gene name, length, coordinates on the *P. luminescens* chromosome, orientation and brief description. Bold type indicates gene interrupted in BMM320

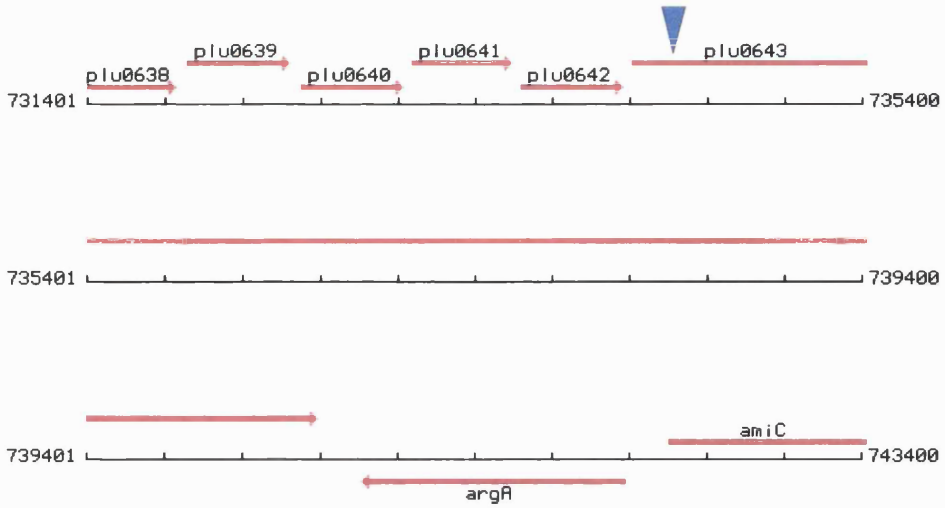


Figure 3.12. BMM321 insertion site and surrounding genes. Blue arrow represents point of insertion. Red arrows represent coding sequences.

Gene	Gene length	Coordinates	Orient-ation	Description
plu0638	522	731335..731856	+	Unnamed protein product
plu0639	531	731915..732445	+	Unnamed protein product
plu0640	522	732504..733025	+	Unnamed protein product
plu0641	522	733074..733595	+	Unnamed protein product
plu0642	522	733644..734165	+	Unnamed protein product
plu0643	**2124**	**734216..740587**	+	**Serine protease, subtilase family**
argA	1347	740819..742165	-	Amino-acid acetyltransferase (N-acetylglutamate synthase)
amiC	1245	742410..743654	+	N-acetylmuramoyl-L-alanine amidase precursor

Table 3.12. Table of genes surrounding insertion site in BMM321. Table includes gene name, length, coordinates on the *P. luminescens* chromosome, orientation and brief description. Bold type indicates gene interrupted in BMM321.

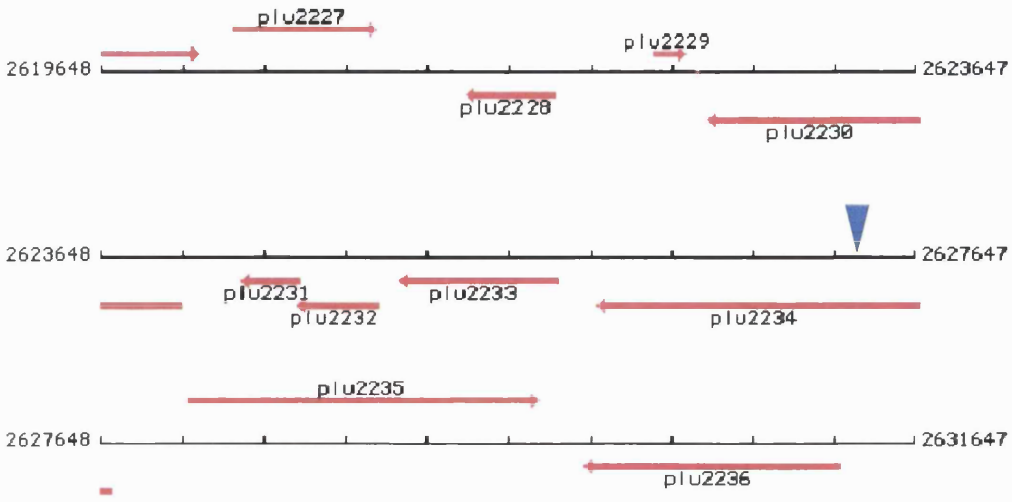


Figure 3.13. BMM323 insertion site and surrounding genes. Blue arrow represents point of insertion. Red arrows represent coding sequences.

Gene	Gene length	Coordinates	Orient-ation	Description
Plu2227	708	2620291..2620998	+	Unknown, probable dethiobiotin synthetase
plu2228	393	2621452..2621844	-	Unnamed protein product
plu2229	156	2622358..2622513	+	Unknown
plu2230	1398	2622631..2624028	-	Hypothetical protein
plu2231	264	2624343..2624606	-	Hypothetical protein
plu2232	363	2624622..2624984	-	Hypothetical protein
plu2233	750	2625116..2625865	-	Unknown, putative oxidoreductase3-oxoacyl-[acyl-carrier protein
plu2234	**1599**	**2626090..2627688**	-	**Histidine ammonia-lyase**
plu2235	1704	2628089..2629792	+	Probable exochitinase
plu2236	412	2630027..2631262	-	Probable FAD-dependent monooxygenase

Table 3.13. Table of genes surrounding insertion site in BMM323. Table includes gene name, length, coordinates on the *P. luminescens* chromosome, orientation and brief description. Bold type indicates gene interrupted in BMM323.

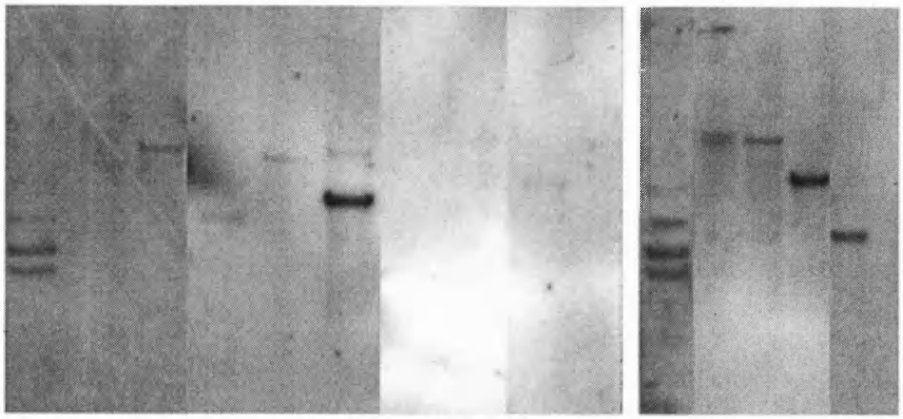


Figure 3.14. Southern Hybridisation of DIG labelled kanamycin resistance cassette probe to *Eco*RI cut genomic DNA from 13 mutants isolated as being defective in motility. M = lane loaded with marker DNA. Lane 1 = BMM304, 2 = BMM305, 3 = BMM307, 4 = BMM308, 5 = BMM309, 6 = BMM313, 7 = BMM314, 8 = BMM316, 9 = BMM317, 10 = BMM320, 11 = BMM321, 12 = BMM302, 13 = BMM323.

3.2.5 Analysis and Discussion of Mutants

3.2.5.1 Group I

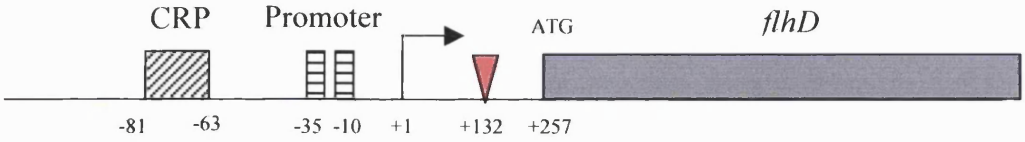
Group I consists of 3 mutants, BMM307, BMM309 and BMM316, which have insertion sites in or near genes with obvious links to motility. BMM307 contained an insert 125bp upstream of the predicted ATG start codon of *flhD*, which is part of the flagella master regulon. Although it is not disrupting the coding sequence of the regulatory gene the phenotype of the mutant is non-motile. The mutant BMM307 also lacks lipase activity, which is similar to the *flhD* mutants in *Xenorhabdus*, suggesting that the mechanisms linking motility to lipase are conserved in both strains. Interestingly BMM307 was also slightly attenuated in virulence (90%), as was the *Xenorhabdus* mutant (approximately 75-80%) (Givaudan and Lanois, 2000). These results and the fact BMM316 is unaffected in virulence suggests that the slight attenuation in virulence of the *flhD* mutants may be due to the lack of lipase activity rather than a lack of motility.

One hypothesis for BMM307 being non-motile is that the transposon may have inserted into a regulatory region upstream of *flhD*. In *Xenorhabdus* there is a putative cyclic AMP (cAMP)-receptor protein (CRP) binding site that starts 341bp upstream of the start codon of *flhD* (Givaudan and Lanois, 2000). In *Photorhabdus* there is a site with homology to the CRP binding sequence that starts 338bp upstream of the putative start codon for *flhD*. This putative CRP binding site for both *Photorhabdus* and *Xenorhabdus* has homology to the *E. coli* CRP binding site (Fig. 3.15 B) (Soutourina *et al.*, 1999). Furthermore, putative –35 and –10 promoter sequences were identified by high homology to known *E. coli* promotor sequences. The –35 hexamer is 50% identical and the – 10 hexamer is 67% identical, for all three strains, to the σ^{70} consensus sequence (Fig. 3.15 C).

The transcription initiation site for *E. coli flhDC* transcript starts 6bp downstream of the end of the –10 sequence and this is approximately 200bp upstream from the ATG start codon (Soutourina *et al.*, 1999). In *Photorhabdus* and *Xenorhabdus* there are predicted to be 255 and 260bp between the putative transcription start sites and the ATG start codons of the *flhD* genes, respectively. The insert in BMM307, which is 125bp upstream of the start codon, would be downstream from the putative transcription start site and would therefore interrupt the *flhDC* transcript (Fig. 3.15 A).

Interestingly, in mutant BMM309 the transposon had inserted 180bp downstream from the predicted stop codon of *fliT*, in a region with homology to an area between the flagella operons of *fliFGHIJK* and *fliDST* (Fig. 3.7). In *E. coli* and *Salmonella* this region contains the single flagella gene *fliE*, and in *Photorhabdus* this area also contains a putative gene with homology to *fliE*. However a second gene (*plu1950*) with unknown function and no homology in the database is also located within this region. In *E. coli* the *fliFGHIJK* operon consists of class II genes and encoded proteins involved in the synthesis of the MS ring, the switch protein, an ATPase involved in flagella export and a protein that controls the length of the hook (Macnab, 2003). The gene *fliE* encodes a class II structural component of the basal body and is probably located at the junction between the

(A) Genomic region surrounding insertion site of BMM307



▼ = Site of insertion, 125bp upstream of *flhD* ATG.

CRP = Putative site of binding for catabolite repressor protein

+1 = Putative transcription start site

(B) Putative CRP binding site

-338 TTGTGAGTTATGTCACATA -320		*Photorhabdus luminescens*
-341 TTGTGATCGGTATCACATA -323		*Xenorhabdus nematophila*
TGTGATCTGCATCACG		CRP consensus sequence

(C) Putative promoter region

-36 TTGTAT – 18bp – TAAAGT -7		*Photorhabdus luminescens*
-36 TTGTAT – 18bp – TAAAGT -7		*Xenorhabdus nematophila*
-35 TTGTAT – 17bp – TACAGT -7		*Escherichia coli*
TTGACA ~ 17bp ~ TATAAT		σ^{70} consensus sequence

Figure 3.15. (A) Diagram of genomic region surrounding the insertion site of BMM307 including the putative *flhD* ATG start codon, transcription start site, promoter region and CRP binding site. (B) Putative CRP binding sites for *Photorhabdus* and *Xenorhabdus*. Homology is highlighted in yellow, homology between *Photorhabdus* and *Xenorhabdus* is highlighted in green. (C) Putative –35 and –10 sequences for *Photorhabdus* and *Xenorhabdus* based on the homology to *E. coli* regions and σ^{70} consensus sequence. Regions of homology between the three strains are highlighted in yellow, between *Photorhabdus* and *Xenorhabdus* are highlighted in green, base pairs shared by all three strains are highlighted in blue.

MS ring and the rod. The operon *fliDST* is a class III operon and consists of genes encoding the filament capping protein (encoded by *fliD*) and two chaperones, one for the filament protein FliC (encoded by *fliS*) and one for the filament capping protein FliD (encoded by *fliT*) (Macnab, 2003). The putative gene, *plu1950*, is 813bp in length and situated 402bp downstream from the last gene in the – orientated *fliDST* operon, reading in the + direction. Furthermore it is 598bp downstream of *fliE*. This data strongly suggests this gene is not part of either the *fliDST* or *fliE* operons. The protein encoded by *plu1950* has no homology to any known protein and its function in motility can only be speculated on. Although the insertion in BMM309 did not directly insert into a gene the insertion of a kanamycin resistance cassette, approximately 1Kb in size, into this flagella biosynthesis region may have affected the transcription of a number of genes in this area. Recently gene products with previous unassigned roles are being characterised as chaperones (encoded by *fliT*, *flgN*), non-essential regulators of other flagella genes (encoded by *fliH*) and capping proteins (encoded by *flgJ*) (Fraser *et al.*, 1999; Nambu *et al.*, 1999; Macnab, 2003). The gene *plu1950* may therefore have a non-essential, but direct, role in motility.

Furthermore, novel genes without roles in motility are being identified as members of the flagella regulon. In *Salmonella*, *fliZ* is located downstream of the sigma factor for late flagella gene expression *fliA* and encodes a positive regulatory factor for genes involved in mammalian cell invasion (Ikebe *et al.*, 1999; Iyoda *et al.*, 2001). The *Yersinia* phospholipase gene *yplA* is not involved in either flagella structure or function; however it is part of the flagella regulon and is transcriptionally regulated by *fliA* (Schmiel *et al.*, 2000). Therefore genes and phenotypes unrelated to flagella can be regulated with motility. Interestingly the symbiont *Vibrio fischeri* has been suggested to genetically regulate motility with symbiosis via the flagellar global transcriptional regulator *flrA* (Millikan and Ruby, 2003). Therefore *plu1950* may have an as yet unidentified role in *Photorhabdus* flagella structure or synthesis or it may be involved in a phenotype regulated with motility.

Finally BMM316 has an insertion in nucleotide 705 of *flgG*, a class II flagella structural gene, encoding a protein that forms the distal portion of the flagella

basal body. This mutant is completely non-motile. The gene *flgG* is part of an operon of 9 genes involved in the construction of the basal body and hook (Fig. 4.1) (Macnab, 2003). It is logical to assume the insertion in BMM316 has polar effects on *flgHIJ* and that the construction of a complete basal body does not occur. Without a complete basal body the rest of the flagella apparatus cannot be constructed and this accounts for non-motile nature of BMM316 (See chapter 4).

3.2.5.2 Group II

During the screen for motility defective mutants BMM305, BMM308 and BMM320 were isolated. These mutants have inserts in genes with putative roles in membrane modifications or that encode proteins likely to be located in the membrane. Bacterial membranes are constructed from a mixture of phospholipids, lipopolysaccharides and proteins and disruptions in membrane homeostasis could lead to disruption of normal flagella production or function.

In BMM305 the transposon disrupted the gene *pbgE1*, which has 45% identity at the amino acid level, to the protein PmrK in *Salmonella* that encodes a membrane protein involved in LPS modifications. The mutant BMM305 is slightly less motile than its parent strain and the putative disruption in the LPS could account for the disruption in motility. Bacterial flagella consist of many proteins that have to span the entire length of the bacterial membrane and each layer of membrane has specific flagella proteins associated with it. The L ring protein encoded by *flgH* in *Salmonella* is inserted into the outer membrane, the hook and filament proteins are external to the outer membrane and are therefore likely contact or interact with O antigen (Macnab, 2003). The lipid A, core and O antigen region have all been implicated as important in interactions with membrane proteins. The *E. coli* outer membrane protease OmpT was over-expressed and isolated; however the enzyme was only active upon addition of LPS (Kramer *et al.*, 2002). Further investigation demonstrated that OmpT requires a fully acylated lipid A and heptose bound phosphates in the inner core region to be active and it was hypothesised that correct LPS causes a conformational change in the enzyme that permits activity (Kramer *et al.*, 2002). Furthermore in *Shigella* correct core and O antigen structure affect the localisation of another outer membrane protein associated with virulence

(Sandlin *et al.*, 1995). Lastly the negative charges in the core region of LPS contribute to the correct folding of the outer membrane pore protein PhoE (de Cock *et al.*, 1999). Therefore LPS has an interactive role with membrane proteins effecting conformation, localisation and folding. It is unknown how the lipid A, core or O antigen region may interact with flagella proteins; however some form of interaction is likely and major structural changes in LPS will probably affect this.

The mutant BMM308 contains an insert approximately 550bp upstream from a serine transporter and 500bp downstream from a putative permease. It is probable that the insert affected the transcription of the serine transporter; however without further analysis it is difficult to be certain. Flagella are metabolically expensive as a single flagella filament can consist of up to 20,000 units of flagellin (Macnab, 2003), which is a significant metabolic burden especially in bacteria with peritrichous flagella such as *Photorhabdus*. A single *Photorhabdus* flagellin protein is 355 amino acids in size and over a third of the protein is made up of just alanine, leucine, asparagine and serine. Therefore, if the transposon insert in BMM308 affects the gene encoding a serine transporter one hypothesis is that the strain may be deficient in serine, which is an extremely important amino acid in flagella production. The mutant BMM320 had an insert just downstream of another putative membrane protein; however the region further contains genes with very little homology to any other known genes and it is difficult to hypothesise a function in motility.

3.2.5.3 Group III

A third group of motility mutants includes strains with inserts in genes whose products putatively affect the metabolism of the cell. It is likely that these mutants are all at a metabolic disadvantage and therefore may not synthesise flagella to the same degree as a parental strain.

The mutant BMM323 harbours a mutation in a gene whose product has 51% identity at the amino acid level to histidine ammonia-lyase (HAL). HAL is the first enzyme in the breakdown of the amino acid histidine, specifically, it removes the α-amino group resulting in the production of trans-urocanate

(Baedeker and Schulz, 2002). Interestingly HAL is closely homologous to phenylalanine ammonia-lyasae (PAL), which is common in plants and is a key enzyme in the production of secondary metabolites (Xiang and Moore, 2002). In *Streptomyces maritimus* this enzyme forms the first step in a biochemical pathway that converts L-phenylalanine to enterocin, an antibiotic (Xiang and Moore, 2002). *Photorhabdus* has operons (*hutIGC* and *hutUH*) involved in the breakdown of histidine including another gene with homology to histidine ammonia lyase (*hutH)*. Therefore it is possible that the gene interrupted in BMM323 may function as a PAL and subsequently is involved in the production of secondary metabolites. In support of this the gene interrupted in BMM323 has 32% identity at the amino acid level to the PAL of *S. maritimus*.

The mutant BMM321 has an insert in a gene encoding a serine protease. This strain may be affected in the initial stages of breaking down larger substrates for metabolism and therefore may have a metabolic defect.

Interestingly, the inserts in the two mutants BMM314 and BMM317 are in two genes separated by approximately 4Kb, although it appears the insertions are in two different operons (Fig. 3.9). The insert in BMM317 is in the last gene of a putative three-gene operon as there are no more than 10bp separating the genes *plu0755, plu0754* and *plu0753*. Approximately 350bp upstream of *plu0755* is *plu0756* (site of insertion in BMM317) this is the last gene is a putative operon containing 10 genes. The identification of two insertions in this locus suggests this area has a role in motility and may be a favourable site of insertion by the transposon. The mutant BMM314 had an insert in a gene with a gene with 31% homology at the amino acid level to a non-ribosomal peptide synthetase, and BMM317 had an insert in a gene with homology to an acyl carrier protein. The products of these genes are enzymes putatively involved in the synthesis of non-ribosomal peptides. Furthermore, BMM302 has an insert in *ngrA*, a gene that encodes a 4'-phosphopantetheinyl (Ppant) transferase, an enzyme further involved in the production of non-ribosomal peptides as well as fatty acid and polyketide synthesis (Ciche *et al.*, 2001).

The gene *ngrA* is most homologous to *entD*; a gene in *E. coli* involved in the production of the siderophore enterobactin and a *Photorhabdus luminescens* NC1 *ngrA* mutant was defective in siderophore production. However, Ppant transferases can be divided into two groups, those involved in modification of fatty acids (AcpS type - approx 120 amino acids) and those with a wide substrate range including non-ribosomal peptides, polyketide synthetase and fatty acid synthetase (Sfp type - approx 240 amino acids) (Mofid *et al.*, 2002). The protein NgrA is 248 amino acids long; therefore it is likely to fall into this second group, furthermore the publication of the *Photorhabdus* genome reveals it has a copy of the *acpS* gene (*plu3336*). Therefore the Ppant transferase encoded by *ngrA* is likely to have wide substrate specificity, and whilst in NC1 it has a role in the production of siderophores, in TT01 it may also have a role in the production of other metabolites. Further to this BMM302 is hyperpigmented, suggesting a role for *ngrA* in pigmentation production.

Surfactins are metabolites produced by non-ribosomal peptide synthetases and are important in both flagella dependent and independent bacterial motility by reducing surface tension and friction and allowing more rapid colony expansion (Toguchi *et al.*, 2000; Kinsinger *et al.*, 2003). It is possible that the lack of motility in BMM314, BMM317 and BMM302 is due to effects on the production of surfactants through non-ribosomal peptide synthetases. An alternative product of the Sfp type of Ppant transferase are fatty acids and interestingly, fatty acid derivatives have been shown to be important in intracellular signalling (Soto *et al.*, 2002; Lucas *et al.*, 2000). Therefore a second putative role for TT01 *ngrA* in motility may be to help produce intracellular signals for initiating motility.

3.2.5.4 Group IV

Two mutants had inserts in genes that did not appear to have direct or indirect links to motility. BMM304 has an insertion in a gene with homology to photopexin and BMM313 has an insert in a gene with homology to an intergrase, which is an enzyme that catalyses the integration of one DNA into another by recombination.

3.2.6 Phenotypic Screens of the Mutants

Motility has been linked with phenotypic variation in *Xenorhabdus* (Givaudan *et al.*, 1995). In order to assess whether motility was linked to phenotypic variation in *Photorhabdus*, and also to determine whether the insertions have pleiotropic affects on the mutants, each mutant was screened for primary variant characteristics. The screens assessed the mutant's ability to produce pigmentation, lipase, protease and bioluminescence (Table 3.14). All the mutants were pigmented (yellow/brown) and no qualitative difference in pigment was observed, except for BMM302 and BMM323, which were hyperpigmented (dark orange/red). All the mutants were able to bioluminesce and all produced proteases; however BMM321 and BMM304 were slightly attenuated in protease production when compared to parental levels. This reduced level in BMM321 supports the genetic evidence that it has an insert in a gene with 55% identity at the amino acid level to a protease. The mutant BMM304 has an insert in a gene with some homology to photopexin, a toxin from *Photorhabdus*, it is also approximately 12 genes away from photopexin A and B indicating that the transposon is in an area associated with toxin expression and virulence. This does not shed light on why BMM304 is less motile than TT01 or why it produces slightly less protease. All the mutants produced lipase to the same degree as the parent strain with the exception of BMM307, which showed greatly reduced levels of lipase production. This is interesting as BMM307 has an insert just upstream of the flagella master regulon *flhDC* and in *Xenorhabdus* a *flhDC* mutant was also defective in lipase activity (Givaudan and Lanois, 2000).

Two mutants, BMM302 (*ngrA*) and BMM323 (PAL), were attenuated in motility were hyperpigmented and also produced slightly higher levels of lipase activity than TT01. Furthermore BMM302 was defective in the production of antibiotics and siderophores (Table 3.15). The gene *ngrA*, which was interrupted in BMM302, has high homology to the *ngrA* gene of *Photorhabdus luminescens* NC1, which has been previously reported as being required for nematode growth and reproduction (Ciche *et al.*, 2001). The production of lipase and pigmentation were observed in the NC1 *ngrA* mutant, but no discrepancies were reported (Ciche *et al.*, 2001). The differences observed may be due to the

Mutant	Gene/product affected	Pigment	Lipase	Protease	Biolum-inescence
TT01		+	++	++	+
BMM302	*ngrA*	++	+++	++	+
BMM304	Photopexin homologue	+	++	+	+
BMM305	*pbgE1*	+	++	++	+
BMM307	*flhD*	+	W+	++	+
BMM308	Serine transporter	+	++	++	+
BMM309	*fliT?/plu1950*	+	++	++	+
BMM313	Intergrase	+	++	++	+
BMM314	NRPS	+	++	++	+
BMM316	*flgG*	+	++	++	+
BMM317	ACP	+	++	++	+
BMM320	*plu1627*	+	++	++	+
BMM321	Serine protease	+	++	+	+
BMM323	HAL	++	+++	++	+

Key. - = negative result. + = positive result. W+ = weakly positive.

Table 3.14. Mutants were assessed for their ability to produce the primary characteristics, pigment, lipase, protease and bioluminescence, as discussed in Materials and Methods.

different strains used. A transposon insert in *Photorhabdus temperata* K122 was also identified in our laboratory as being in *ngrA* (J. Williams, personal communication.) (For a tabular comparison of TT01 K122 and NC1 see table 3.15). This strain was affected in pigment production; K122 on LB agar appears yellow, K122 *ngrA* appears more orange. However like NC1, K122 *ngrA* appears normal for lipase production (J. Williams, personal communication).

Phenotype	**TT01 *ngrA***	**NC1 *ngrA***	**K122 *ngrA***
Motility	+/-	ND	+
Pigment Parent Pigment Mutant	Orange/brown Bright red/orange	No difference observed.	Yellow Orange
Lipase	++	+	+
Protease	+	+	+
Antibiotic	-	-	-
Siderophore	-	-	+/-
In vitro symbiosis	+	-	ND
In vivo symbiosis	- (HD)	N/A	ND
Source	This study	Ciche and Ensign (2001).	J. Williams. Personal Communication

Key = + = equivalent to parental strain. - = defective compared to parental strain. ++ = excess compared to parental strain. +/- = weak compared to parental strain. HD = Highly defective. ND = Not done. N/A = not applicable

Table 3.15. Comparison of primary characteristics of *ngrA* mutants of *Photorhabdus luminescens* TT01, *Photorhabdus temperata* K122 and *Photorhabdus luminescens* NC1.

Interestingly there are subtle differences between the phenotypes of the *ngrA* knockouts of NC1, TT01 and K122, which may be due to slightly different substrate specificity or products generated by the *ngrA*-encoded Ppant transferases.

Polyketide synthases are involved in the production of secondary metabolites, one of which may be pigment. Therefore *ngrA* may be biochemically linked to pigment production. The NC1 *ngrA* mutant was deficient in antibiotic and siderophore production as was the TT01 *ngrA*. However, the K122 *ngrA* was deficient in antibiotic production and attenuated in siderophore production

indicating a similar biosynthetic end point for antibiotic and siderophore production, and a similar role for *ngrA* in this pathway in all three strains.

The mutant BMM323 had an insert in a gene with homology to HAL and was also hyperpigmented and hyper for lipase production. Although, as discussed above, it is likely that the HAL gene interrupted may be a PAL gene. As PAL's are involved in the production of secondary metabolites (Xiang and Moore, 2002) this gene may have a role in the hyper production of pigment and lipase in TT01. One hypothesis is PAL could be involved in the breakdown of a substrate for pigment production; therefore a knockout of PAL would lead to an excess of substrate for the pathway. These results indicated the transposon insertions did not have significant pleiotropic effects and, that although individual mutants may be altered in some primary characteristics, overall there is no direct link between the ability to swim with the primary phenotypic characteristics of TT01.

3.2.7 Symbiosis Screens.

The motility mutants were then screened for the ability to support nematode growth *in vivo* and *in vitro* (Table 3.16). The two mutants most severely affected in swimming motility were BMM307 and BMM316, which were able to support nematode growth and development to the same level as TT01. Further to roles in siderophore, antibiotic and pigment production and motility in TT01, a knockout in *ngrA* (BMM302) affects the ability of *Heterorhabditis* to grow and develop on TT01. Interestingly, BMM302 supported the *in vitro* growth of the nematode partner, contrary to the findings of Ciche and Ensign (2001). However, after infection of inset larvae by nematodes containing BMM302, qualitatively fewer nematodes were returned after *in vivo* growth and development. These results lend further support to the hypothesis that *ngrA* in TT01 has a subtly different role to *ngrA* in NC1. It has been shown that siderophores and motility are not essential for symbiosis (Ciche *et al.*, 2003). It is therefore likely the *ngrA* Ppant transferase has additional roles in generating symbiotic signals, further demonstrating the wide range of substrates used and products generated. The importance of *ngrA* is evident due to the fact that it was isolated in three independent screens trying to identify factors important in symbiosis (NC1), antibiotic production (K122) and motility (TT01).

Strain	Gene/product affected	Symbiosis *in vitro*	Symbiosis *in vivo*	Virulence (injection)	Virulence (infection)
TT01		+++	++	100%	100%
BMM302	*ngrA*	+++	+	100%	100%
BMM304	Photopexin homologue	+++	++	90%	100%
BMM305	*pbgE1*	+++	-	40%	0%
BMM307	*flhD*	+++	++	90%	100%
BMM308	Serine transporter	+++	++	100%	70%
BMM309	*fliT?/plu1950*	+++	++	100%	90%
BMM313	Intergrase	+++	++	100%	70%
BMM314	NRPS	+	ND	100%	ND
BMM316	*flgG*	+++	++	100%	90%
BMM317	ACP	+++	++	100%	90%
BMM320	*plu1627*	+++	++	100%	100%
BMM321	Serine protease	+++	++	100%	100%
BMM323	HAL	+++	++	100%	90%

Key. - = negative result. + = positive result.

Table 3.16 Table of motility mutants. Each mutant was briefly assessed for its ability to support symbiosis with *Heterorhabditis bacteriophora* and its virulence towards *Galleria mellonella*. NRPS = Non ribosomal peptide synthase. ACP = Acyl carrier protein. These are the results of one screen.

BMM314 was the only mutant that qualitatively appeared to be attenuated in *in vitro* nematode growth and development and insufficient IJs were collected from BMM314 to infect insect larvae and assess pathogenicity by infection and *in vivo* symbiosis. The insert in this mutant was identified as being in a putative non-ribosomal peptide synthetase. Interestingly, BMM317, which contains a mutation

in an acyl-carrier protein only 2.5Kb downstream from BMM314, was unaffected in symbiosis. Acyl carrier proteins require phosphopantetheinyl transferases (*ngrA*) for some of their activities and are involved in the biosynthesis of fatty acids, polyketides and non-ribosomal peptides. Although this suggests putative involvement in a similar biochemical pathway between BMM317 BMM314 and BMM302, BMM317 displayed no symbiosis defect.

All other mutant strains appeared to support *in vitro* nematode growth and development. However when nematodes grown on BMM305 were used to infect *Galleria mellonella* larvae the insect larvae did not die and no IJs emerged, which suggests that BMM305 may be attenuated for *in vivo* nematode growth and development (See chapter 5).

3.2.8 Pathogenicity Screens

The two strains most affected in motility were BMM316 and BMM307 and both of these strains appeared as pathogenic as TT01 (Table 3.16). Indeed all the mutants were able to kill *Galleria mellonella* to an extent when injected directly, however BMM305 appeared to be attenuated in virulence by injection (Table 3.16). Furthermore, all mutants except BMM305 were pathogenic in association with the nematode host. These results indicate that motility *per se* does not appear to be linked to the pathogenicity of TT01. These results also suggest that the putative protease interrupted in BMM321 and the gene with homology to photopexin interrupted in BMM304 are not virulence factors.

3.3 Conclusion.

Photorhabdus luminescens TT01 is a motile bacterium that can swim, swarm and twitch. Our objective was to determine whether motility, a primary specific phenotype, could be correlated with either pathogenicity or symbiosis. We have screened approximately 3000 mutants for swimming defects and our results suggest that there is no correlation between motility and either symbiosis or pathogenicity. In addition we have screened approximately 4000 exconjugants for twitching defects, but no twitching mutants were isolated. However since the screen was undertaken the publication of the TT01 genome has facilitated *in*

silico analysis of the genome and this has yielded two putative *pil* operons (Duchaud *et al.*, 2003). This redundancy may explain why twitching mutants were difficult to isolate.

P. luminescens TT01 has 48 genes (Fig. 1.0) with high homology to genes involved in motility in other bacteria, including the master regulon *flhDC*. The screen for swimming motility defects in TT01 was intended to reveal mutants with inserts in genes associated with flagella production and function. However the screen compared motility of the mutants by means of a halo around an initial point of inoculation. Therefore mutants that were slower swimmers for a number of different reasons were also identified in this screen leading to the characterisation of novel genes with undefined roles in motility.

Approximately 3000 mutants were screened and 3 mutants were identified that had inserts in or near characterised flagella genes; therefore approximately 1 in 1000 exconjugants (0.1% mutation rate) were affected in characterised motility genes. The genome of *P. luminescens* TT01 consists of approximately 5,500 genes (Duchaud *et al.*, 2003) and a 0.1% mutation rate indicates approximately 55 genes directly involved in motility. Genomic analysis indicates *Photorhabdus* contains 48 genes with homology to characterised flagella genes indicating this process of mutagenesis and screening is valid. Further mutants with inserts in flagella genes were not isolated as the number screened was too low. Moreover the screen was not stringent and mutants that appeared intermediately defective were isolated giving rise to the second, third and fourth groups of motility mutants as discussed.

Motility is a characteristic of *Photorhabdus* and *Xenorhabdus* that has been associated with phenotypic variation (Givaudan *et al* 1995; Hodgson *et al.*, 2003). It appears from the screens performed in this chapter that motility *per se* is not directly linked to other phenotypic variation characteristics such as bioluminescence, pigment, or protease production; however lipase production does appear to be associated with motility. The mutant BMM307 has a transposon inserted 125 nucleotides upstream of the putative start codon of *flhDC*, and it is almost completely non-motile and also severely attenuated in

lipase activity (Table 3.14). Recent data suggests that the *Yersinia enterocolitica* flagella regulon controls the expression and secretion of *yplA* (Young *et al.*, 1999a; Young *et al.*, 1999b). It is known that the lipase of *Photorhabdus* is not secreted through the flagella export apparatus (Wang and Dowds, 1993). Therefore the evidence from both *Xenorhabdus* and *Photorhabdus flhD* mutants suggest that the flagella regulon may control the expression of the lipase gene.

Although there was no direct link between motility and the primary phenotypic characteristics there may be a link between the production of some secondary metabolites and motility. Four individually isolated motility mutants have inserts in genes that appear to have roles in similar biochemical pathways leading to the production of various secondary metabolites (*ngrA*, HAL/PAL, non-ribosomal peptide synthetase and an acyl carrier protein). One hypothesis is that there is a secondary metabolite that is important for swimming motility, perhaps acting as signal or a surfactant. Swim agar is a semi-solid media and it is possible the production of a surfactant alters the properties and interactions with the water within the agar allowing the cells to move further or faster.

The motility mutants were also assayed for their ability to support nematode growth and development. All mutants could support the development of the nematode partner to some degree, although no correlation between motility and symbiosis could be drawn. The mutants were also assayed for virulence and all except BMM305 were as pathogenic as TT01 to *G. mellonella* larvae. The reasons for the avirulent nature of BMM305 will be detailed in chapter 5.

Interestingly, motility, siderophore, antibiotic and pigment production and importantly symbiosis, are all primary characteristics of *Photorhabdus* and are all linked to *ngrA*, which suggests that the *ngrA* Ppant transferase is an important enzyme in the production of primary characteristics. Moreover lipase production, which has been linked to pathogenicity, is over-produced in a TT01 *ngrA* mutant suggesting an inverse regulation of the factors important in symbiosis and pathogenicity.

In conclusion the mutants BMM316 and BMM307 were most severely affected in motility and these mutants were not affected in either symbiosis or pathogenicity. Therefore motility *per se* did not seem to have a significant role in the life cycle of *Photorhabdus*. However flagella are metabolically expensive to synthesis and utilise. Therefore in order to further analyse the role of flagella in the life cycle of *Photorhabdus* the mutant BMM316 was selected for more stringent examination.

CHAPTER 4

4.0 The *flgG* Gene in *Photorhabdus* Affects Motility and the Colonisation of *Heterorhabditis*.

4.1 Introduction.

Photorhabdus and *Xenorhabdus* have peritrichous flagella and are motile through swimming and swarming and *Photorhabdus* are also able to twitch (see chapter 3) (Givaudan *et al.*, 1995; Hodgson *et al.*, 2003). Transposon mutagenesis and a screen for swimming defects yielded 13 mutants with varying degrees of motility in swim agar. From these mutants it was concluded that motility *per se* did not have a role in the life cycle of TT01. As flagella are metabolically expensive they need to offer the bacteria a selective advantage in order to be retained (Macnab, 2003). Therefore if motility is not essential for the life cycle of *Photorhabdus*, perhaps motility and flagella have a subtler role. This hypothesis is addressed in this chapter by selecting a completely non-motile mutant that is also aflagellate and characterising it further.

For the majority of the motility mutants the lack of motility in swim agar was not due to a direct effect on flagella production, such as the second and third class of mutants discussed in the previous chapter. However, three mutants had inserts in or near genes known to be involved in flagella production or function. The insert in BMM307 was upstream of the flagellar master regulator and, although the strain was non-motile, the insert appeared to have some pleiotropic effects (See chapter 3). The insert in BMM309 was not in a specific gene and, although it was in a locus involved in motility, its effects were difficult to expound. In addition BMM309 was only partially attenuated in motility. The mutant BMM316 was the best candidate for further studies into the role of flagella in the life cycle of *P. luminescens* as it appeared to be completely non-motile. Furthermore the insert is in a structural flagella gene, in a well-characterised operon and the mutation appeared to be non-pleiotropic. Therefore BMM316 was characterised further and used to investigate the role of flagella in the life cycle of *P. luminescens*.

4.2. Results

4.2.1 BMM316 is Disrupted in *flgG* and is Aflagellate

After 48 hours incubation at 28°C on swim agar BMM316 remained completely non-motile growing as a single defined colony (Fig. 4.0). The transposon insertion site of BMM316 was identified as being in nucleotide 705 of a 783bp gene that is 77% identical, at the amino acid level, to the flagellar basal-body rod protein (FlgG) of *Salmonella typhi* (Table 3.1). In *Salmonella* and *E. coli flgG* is part of a class II operon containing 9 genes, *flgBCDEFGHIJ*. This organisation appears to be the same in *P. luminescens* TT01 with the *flgB-J* genes sharing 67%, 65%, 56%, 53%, 63%, 77%, 64%, 68% and 56% identity at the amino acid level to the *flgBCDEFGHIJ* genes of *Salmonella* respectively (Fig. 4.1). The majority of these genes encode well-described structural proteins that together make up the basal body and hook of the flagella (Macnab, 2003). *In silico* analysis has revealed TT01 has 48 genes with annotated homology to the flagella genes found in *Salmonella* and *Escherichia coli* (this study).

Furthermore, these genes are organised in operons with high homology to the flagella operons of *E. coli* and *Salmonella* (Fig. 1.0). Therefore it was expected that BMM316 would not produce flagella. To confirm this transmission electron microscopy was performed on both TT01 and BMM316 using staining specific for visualising flagella. In addition to numerous broken flagella fragments, due to shearing during the preparation, peritrichously arranged flagella were observed on the surface of the TT01 sample (Fig. 4.2). However, all BMM316 cells observed were aflagellate and there was no evidence of broken or fragmented flagella in the medium (Fig. 4.2). Together with the complete absence of motility in swim agar and the nature of the genes disrupted by the insertion it can be concluded that BMM316 is non-motile due to a lack of flagella.

4.2.2 BMM316 is Primary-Like in All Other Characteristics

Photorhabdus can occur in two phenotypic variations, a primary variant and a secondary variant. The primary form is isolated from the nematode and upon

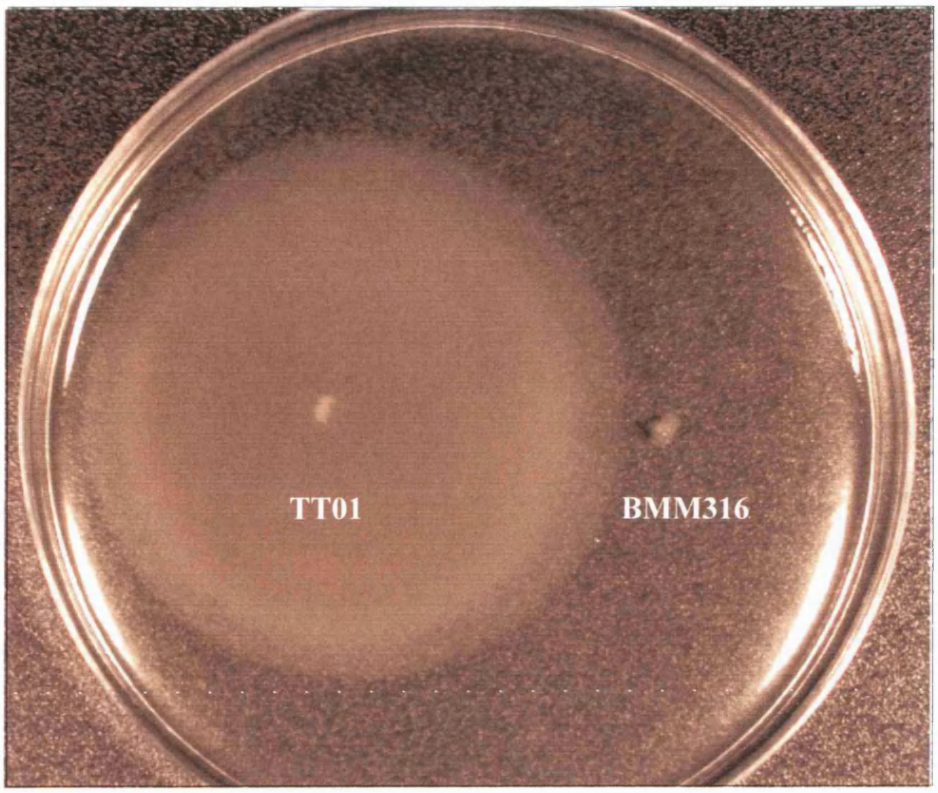


Figure 4.0. 0.3% swim agar plate inoculated with 3µl of overnight culture resuspended to an OD_{600} 1.0, from *P. luminescens* TT01 and BMM316 and incubated at 28°C for 48hrs. The small spots indicate surface colony growth at site of inoculation; the opaque halo around the *Photorhabdus* TT01 site of inoculation indicates swimming cells.

prolonged *in vitro* culturing generates spontaneous secondary variants. The primary variant produces exoenzymes and toxins as well as antibiotics, pigments and light, the primary variant also uptakes certain dyes in agar, is motile and supports nematode growth and production (Akhurst, 1980). These characteristics are either absent or significantly reduced in the secondary variant, and importantly the secondary variant does not support the growth and development of the nematode partner (Akhurst, 1980). The mutant BMM316 has a kanamycin resistance gene inserted in an operon encoding genes with strong homology to structural flagella genes and is non-motile. To assess whether this insertion has pleiotropic effects on any other primary characteristics, tests on these phenotypic

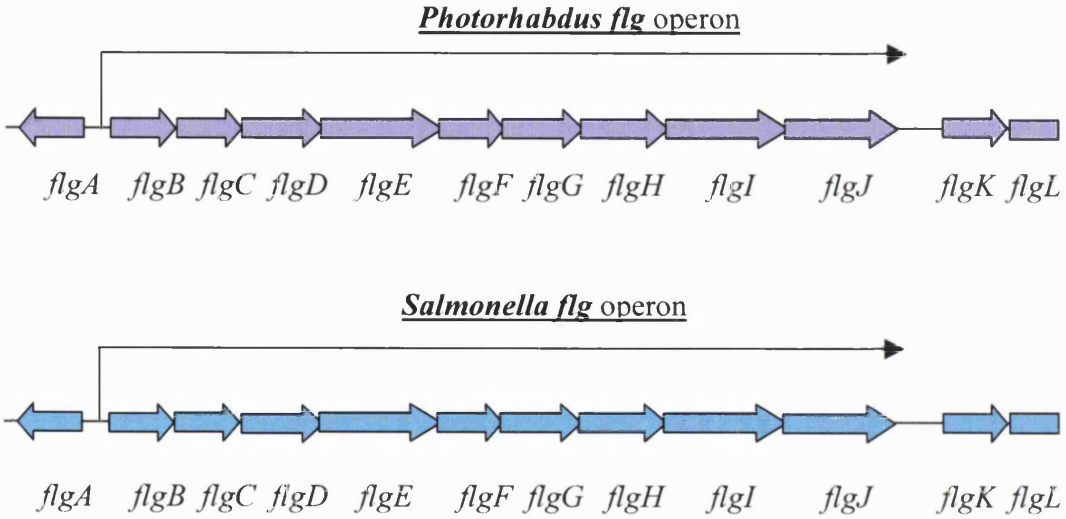


Figure 4.1. Comparison of the *Photorhabdus* and *Salmonella flg* operon using BLASTX, including gene product, cellular location surrounding genes and % identity at the predicted amino acid level

Gene name in *Salmonella*	**Gene product function in *Salmonella***	**% ID to *Salmonella***
flgB	Rod protein	67%
flgC	Rod protein	65%
flgD	Hook capping protein	56%
flgE	Hook protein	53%
flgF	Rod protein	63%
flgG	Distal rod protein	77%
flgH	L-ring protein	64%
flgI	P-ring protein	68%
flgJ	Rod capping protein/ muramidase	56%

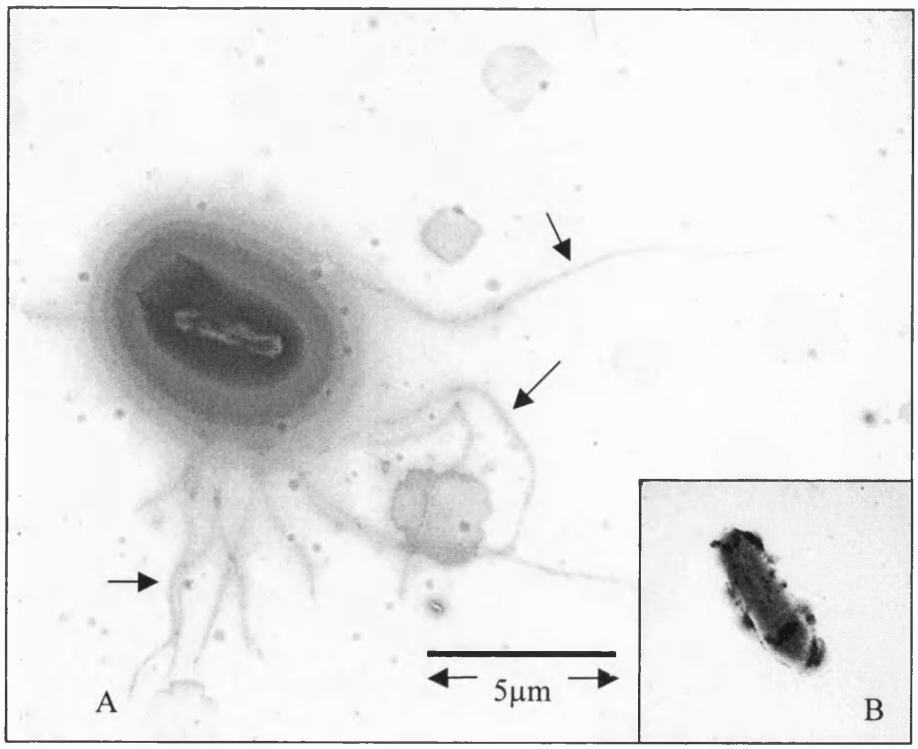


Figure 4.2. Electron microscopy of A) *Photorhabdus luminescens* TT01 and B) BMM316. Bacteria were negatively stained for flagella. Figure A shows the presence of flagella, as indicated by arrows. Figure B shows the absence of flagella around BMM316. Bar represents 5 μm.

characteristics were carried out. BMM316 was shown to be primary like in all the phenotypic tests performed and only differed in motility (Table 4.0). Thus any differences in pathogenicity towards insect larvae or symbiosis with *Heterorhabditis* can be concluded to be the result of BMM316 being aflagellate.

4.2.3 BMM316 Supports Nematode Growth and Development

P. luminescens is the symbiotic partner of the nematode *Heterorhabditis bacteriophora*. *Photorhabdus* is able to support the growth and development of the nematode by providing a food source via the conversion of insect host tissues into a food substrate for the nematode. The symbiosis with the nematode is dependent on two stages. First bacteria must support recovery from the IJ stage

Phenotype		TT01	BMM316
Dye absorption	Mc Conkey	+	+
	EMB	+	+
	NBTA	+	+
Bioluminescence		+	+
Catalase		+	+
Crystalline inclusion proteins		+	+
Extracellular products	Lipase	+	+
	Protease	+	+
	Antibiotics	+	+
	Siderophores	+	+
Motility		+	-
Biofilm formation		+	-
Colony morphology on LB		Round, mucoid	Round, mucoid
Pigmentation on LB		Yellow/brown	Yellow/brown

Table 4.0. Phenotypic tests and the results for *Photorhabdus luminescens* TT01 and BMM316. + indicates a positive result, - indicates a negative or severely reduced phenotype.

into hermaphrodites and successive rounds of reproduction, growth and development. Secondly, the bacteria must be able to successfully recolonise the emerging IJs to enable further insect infections. In order to study the effect motility may have on the growth and development of the nematode partner BMM316 was first grown *in vitro* on lipid agar plates for 48 hours. Approximately 20 surface sterile *Heterorhabditis* IJ nematodes were then added onto these plates, incubated at 25°C and the plates were monitored every second day. Qualitative analysis indicated that the IJs on the plates inoculated with BMM316 recovered to hermaphrodites to the same degree as the IJs grown on TT01 (data not shown). These hermaphrodites laid eggs that developed into males and females and after 3 weeks of development and reproduction large

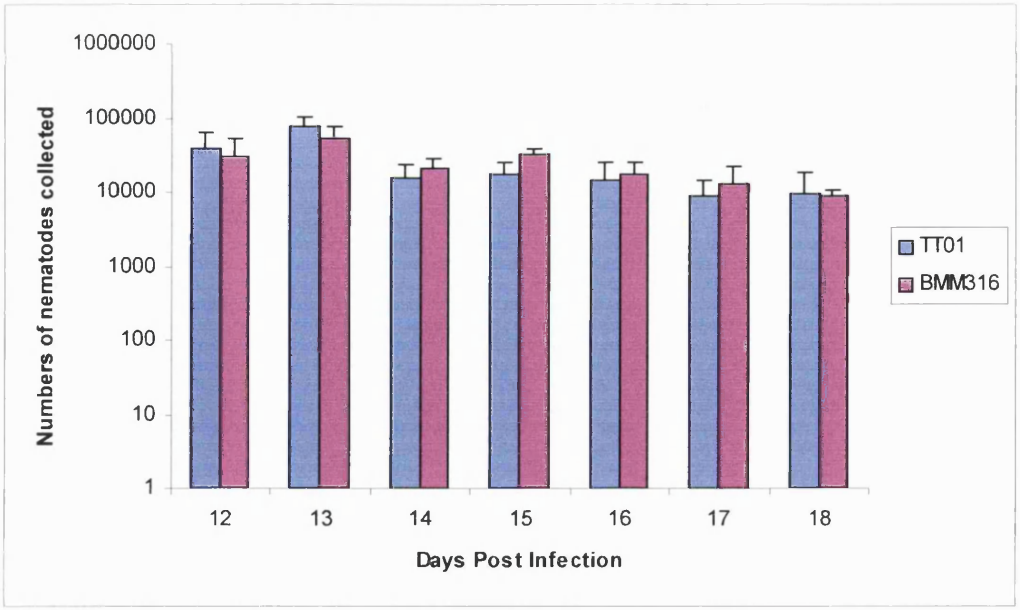


Figure 4.3. Average numbers of IJs emerging from *Galleria mellonella* cadavers, after original infection with IJs carrying either *Photorhabdus luminescens* TT01 (blue) or BMM316 (dark red), over a period of 9 days. Day of infection with IJs = day 0. Bars represent an average of 10 cadavers and Y error bars represent standard deviation.

numbers of IJs started to form and migrate. The time this took to occur was approximately the same for both mutant and TT01 in 5 repeats, and the numbers of IJs migrating were also approximately the same indicating that bacterial flagella are not required for the development of the nematode *in vitro* (data not shown). These IJs were then collected, surface sterilised and allowed to naturally infect *G. mellonella* to assess whether motility affected the growth and development of the nematode partner *in vivo*. The sets of insects infected with TT01 and BMM316 both died within 48 hours, indicating motility is not essential for regurgitation of the bacteria by the nematode. On day 12 post infection the insects were placed on white traps and the emerging IJs collected and counted. As is shown in Fig. 4.3 there was no significant difference in either the time of IJ emergence or the amount of IJs that emerged from the cadavers (Average total TT01 = 182,500. BMM316 = 178,700). These results suggest that flagella have no role in nematode growth or development *in vivo* or *in vitro*.

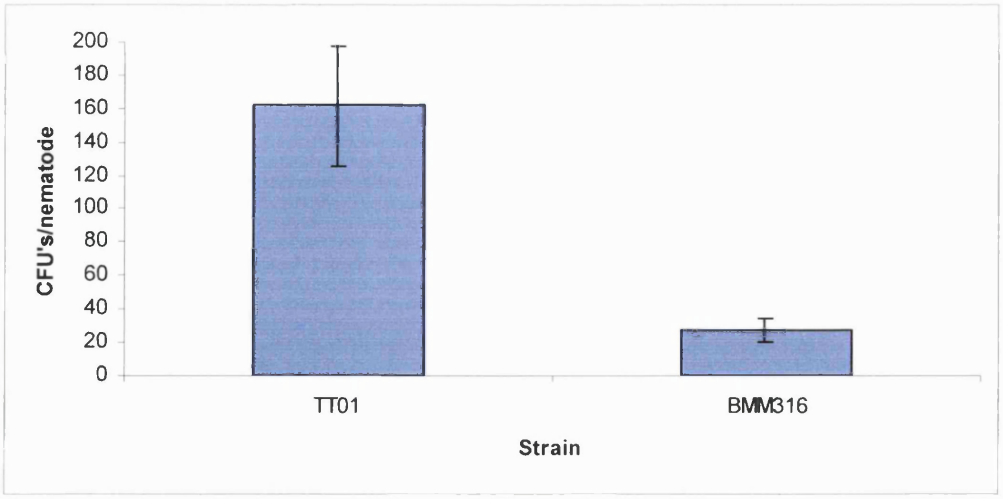


Figure 4.4. Average number of colony forming units (CFU's) of *Photorhabdus luminescens* TT01 and BMM316 obtained per nematode after growth and reproduction on each respective bacterial strain. Number of repeats as follows: TT01 = 20, BMM316 = 15. The Y error bars represent standard deviation.

4.2.4 BMM316 Can Recolonise the Nematode Gut

The second stage of symbiosis as defined above is the ability of *Photorhabdus* to re-associate with the IJ nematode partners as they emerge after growth and reproduction. This is determined by crushing IJ nematodes after migration away from the bacteria (as soon as they emerge onto the petri dish lid) and plating out onto selective media. Both BMM316 and TT01 were seeded onto lipid agar and 1,000 emerging IJs grown on each strain were then surface sterilised and crushed. Nematodes grown on BMM316 were shown to contain approximately 25 cells per nematode whilst nematodes cultured on TT01 were shown to contain approximately 160 cells per nematode (Fig. 4.4). Therefore BMM316 is able to colonise the nematode at only 16% TT01 parental level. This lower level of colonisation is statistically significant with a p value from an unpaired T test of <0.01. However it does not appear to affect further natural insect infection by BMM316 carrying nematodes as experiments have shown these nematodes infect *G. mellonella* and the insects die within 48 hours (Section 4.2.3). Therefore although flagella are important in colonisation of the nematode partner they are not essential.

4.2.5 BMM316 Has a Competitive Disadvantage When Colonising Nematodes

Although the crushing of monoxenic nematodes gives reproducible ratios from strain to strain; the variance in crushings of one strain is quite large from experiment to experiment. Recent data suggests that *Photorhabus* goes through cycles of growth inside the nematode (R. Watson, personal communication) and this could account for this variance. Competitive assays were hence devised to detail the subtle interactions of parental strains and mutant strains within the nematode. These assays would give a final ratio present in the nematode rather than a final total figure. Both BMM316 and TT01 were seeded together in a 1:1 ratio onto 15 lipid agar plates in a symbiosis competition assay and grown for 48h at 28°C. Approximately 20 IJs were added to the plates and incubated at 25°C until IJs started to emerge 3 weeks later. The IJs were crushed and plated out onto selective media to determine the ratio of bacteria present. This ratio is calculated as the number of BMM316/TT01 CFUs and is expressed as a competitive index (CI). A value above 1 indicates a higher number of BMM316 CFUs and a value below 1 indicates a higher number of TT01 CFUs present. The median ratio of bacteria present on the lipid agar plates at time of IJ formation was established (the ratios were determined using both antibiotic resistance markers and motility agar) as 4.79, which indicated that there were many more BMM316 CFUs present than TT01 after three weeks incubation at 25°C. This could be due to several reasons, BMM316 may be better able to survive long periods of incubation because they do not synthesise the metabolically expensive flagella. Alternatively the nematodes may be selectively taking up motile *Photorhabdus* to digest leaving a larger proportion of BMM316 on the LA plate at the time of re-association.

Despite the ratio indicating a larger number of BMM316 CFUs present on the LA plate, the median CI present in the IJs at the time of formation was 0.075, which indicated a higher level of TT01 present in the nematode than BMM316. To take into account the ratio of bacteria on the plate, and therefore to obtain a more accurate CI, the ratio present in the nematodes is divided by the ratio present on the LA plates at the time of re-association. The true median

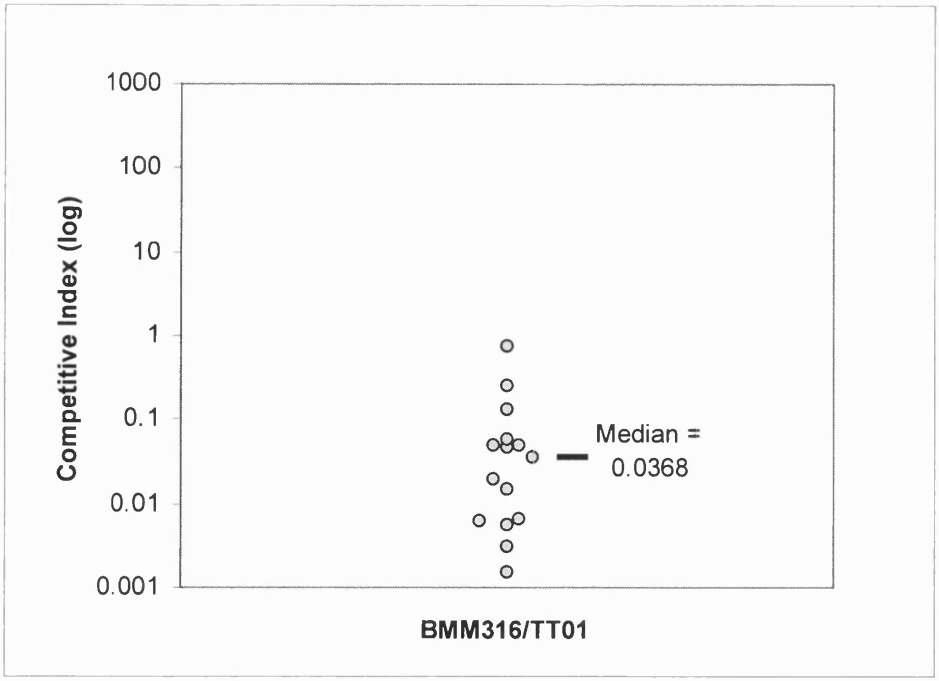


Figure 4.5. *In vitro* competitive index (C.I.) of BMM316/TT01. Each point represents the ratio obtained from nematodes from individual lipid agar plates. The median C.I. for the 15 sets of nematodes was 0.0368.

competitive index ratio is therefore 0.0368 (Fig. 4.5). The data points all clustered below 1 and the majority clustered between 0.1 and 0.01. These results suggest a large number of TT01 present in the nematode compared to BMM316. Therefore flagella are not essential for either growth and development of the nematode or for successful colonisation and completion of the life cycle. However flagella do offer a significant advantage during competitive colonisation of the nematode gut.

Recently Ciche *et al.*, (2003) reported that *P. luminescens* mutants carrying a GFP expressing transposon were affected in the ability to colonise nematodes independent of the site of insertion and they suggested that this might be due to constitutive expression of the GFP. As *in vitro* BMM316 is exposed to kanamycin in order to maintain selective pressure to keep the mutation it is likely that the expression of the kanamycin resistance cassette is constitutive, hence the

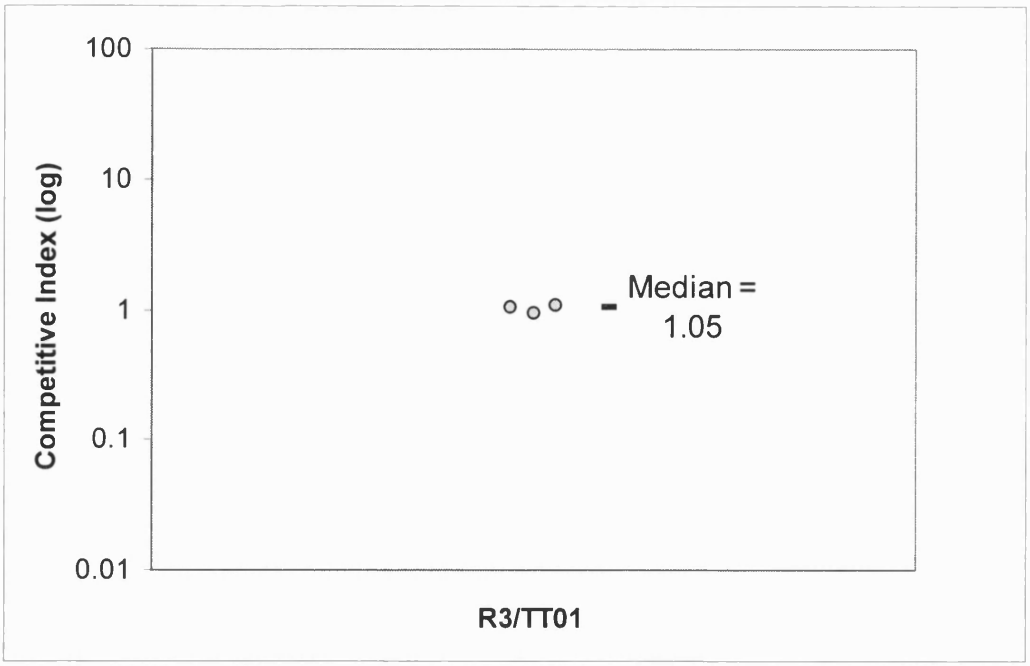


Figure 4.6. *In vitro* competitive index (C.I.) of R3/TT01. Each point represents the ratio obtained from crushing nematodes from individual lipid agar plates. The C.I. for each set of nematodes is calibrated to include the relative ratio of bacteria on the lipid agar plate at time of formation. The median value is 1.05.

kanamycin cassette may have an effect on the competitive fitness and ability of exconjugants to colonise *Heterorhabditis*. In order to test the effects of the kanamycin cassette on BMM316 a mutant (RK3) was chosen at random from a S-17/TT01 conjugation and was competed against TT01 for the colonisation of the nematode partner, as described above. The relative competitive index was approximately 1.0 (Fig. 4.6). This indicates that the kanamycin cassette does not affect the ability of a mutant strain to compete for colonisation. Therefore the competitive disadvantage of BMM316 is due to the motility defect and not a detrimental effect of the kanamycin cassette.

4.2.6 BMM316 is as Virulent as TT01

Photorhabdus cells are highly virulent to insect larvae and less than 5 cells are able to kill 50% of injected larvae of the insect *Galleria mellonella* within 48hrs (data not shown). Motility may play a part in this virulence; for example being

motile may allow the cells to leave unfavourable areas of the insect body or migrate towards nutrient sources or specific areas of colonisation. In addition the flagella themselves are important physical structures on the surface of the bacterium and they may facilitate adhesion to host tissues. To examine the effect of a non-motile aflagellate mutant on virulence towards insects 100 CFUs of TT01 and BMM316 were injected into *G. mellonella*. At this dosage TT01 and BMM316 were lethal to the injected insects within 48hrs. To examine the time of insect death more closely 10 *G. mellonella* insect larva were injected and the insects were examined every hour to determine exact time of death to give an LT_{50} (the time it takes half the insects injected to die). The LT_{50} for TT01 was calculated at 40 $\pm$ 0.5 hours and the LT_{50} for BMM316 is not significantly different from TT01 at 40.5 $\pm$ 1.0 hours. To assess the LD_{50} (the dose at which 50% of the insects injected die) for BMM316 a range of CFUs, from, 1 to 40, were injected into *G. mellonella.* The LD_{50} was less than 5, which is not significantly different from TT01. *G. mellonella* cadavers were surface sterilised and dissected to confirm that BMM316 had not excised the transposon under *in vivo* selective pressure. Colonies of BMM316 were recovered, plated onto swim agar and were shown to remain kanamycin resistant and completely non-motile. In conclusion, these results suggest that a lack of motility does not significantly affect the virulence of *Photorhabdus luminescens* towards *G. mellonella* larva.

4.2.7 Pathogenicity Competition

Motility does not have an essential role in the pathogenicity of *Photorhabdus luminescens* TT01. Therefore in order to determine any subtle role of motility in virulence BMM316 and TT01 cells were mixed in a 1:1 ratio and 100 CFUs were injected into *Galleria mellonella* in order to assess the Competitive Index (CI) of the non-motile bacteria. The insects were surface sterilised at time of death, dissected to release the bacterial cells and the mix of cells was then plated out onto selective media and expressed as a BMM316/TT01 ratio. The median competitive index for 32 insects injected was 0.79 with a wide, but relatively equal, range over 2 orders of magnitude (Fig. 4.7). This reflects the fact that individual insects represent independent environments and the course of TT01 infections depend on each individual insect's health, size, age and immune

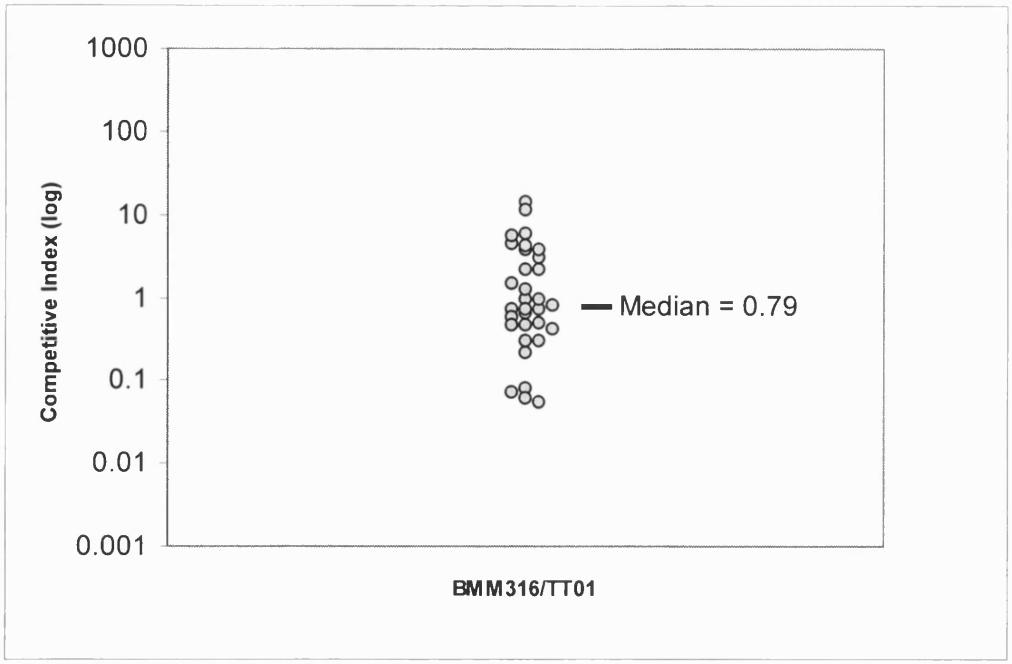
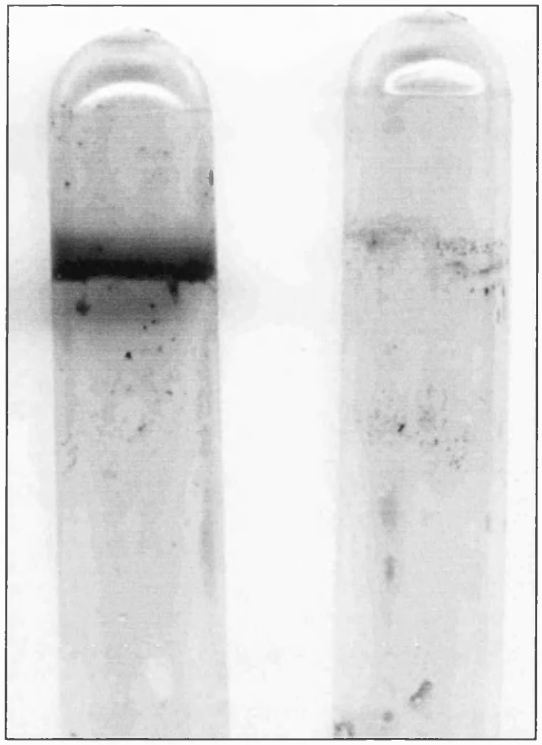


Figure 4.7. *In vivo* competitive index (C.I.) of BMM316/TT01. Each point represents the ratio obtained from an individual insect (n = 32). The C.I. for each insect is calibrated to include the relative ratio of bacteria injected for that experiment (1.66, 2.2, 1.38 and 0.75). The median C.I. for the 32 insects injected was 0.79.

response. The clustering of the data points around a ratio of 1, or just below, indicates an approximately equal number of BMM316 to TT01 cells present at the time of insect larva death. In conclusion these results and the LT_{50} and LD_{50} for BMM316 suggest flagella mediated motility does not play a significant role in virulence.

4.2.8 BMM316 Does Not Form Biofilms

In other bacteria it has been shown that flagella are important in biofilm formation (O'Toole *et al.*, 2000; Watnick *et al.*, 2001). In order to establish the role of flagella in *Photorhabdus* biofilm production BMM316 and TT01 were inoculated into polypropylene tubes containing LB broth. The tubes were left without shaking at 25°C for 48hrs before staining with crystal violet (Fig. 4.8). Under these conditions TT01 produced a biofilm as shown by the presence of a



(A) (B)

Figure 4.8. Biofilm formation of *Photorhabdus* in polypropylene 5ml culture tubes. Biofilms are stained with 1% (w/v) crystal violet. Tube (A) shows the biofilm formed by *Photorhabdus luminescens* TT01 and tube (B) shows the biofilm formed by BMM316 after 48hrs static incubation at 25°C.

clear purple band. On the other hand BMM316 appears to be defective in biofilm formation, with the crystal violet staining only in small pits, suggesting flagella are important in TT01 biofilm formation. However if incubated for longer (a further 24 hours at 25°C) BMM316 starts to show the formation of a biofilm band suggesting that although flagella may be important for initial interaction and attachment to surfaces this can be overcome by high cell densities (Lawrence *et al.*, 1987; O'Toole and Kolter, 1998).

4.3. Conclusion

Studies have shown poor survival rates for *P. luminescens* in soil (Morgan *et al.*, 1997), and as yet *P. luminescens* cells have not been isolated from either soil or water suggesting that *Photorhabdus* spends its whole life cycle associated with either insect or nematodes hosts. Bacterial-host interactions can be mediated through signalling molecules and through structural appendages; including flagella, fimbriae, pili and LPS.

The role that flagella mediated motility has in the life cycle of *Photorhabdus* is unclear. Recent work by Givaudan and Lanois (2000) involving a *Xenorhabdus flhDC* non-motile mutant suggested that motility might be important in virulence. However FlhD has functions outside of motility as it has been found to regulate cell division in *E. coli* and phospholipase excretion in *Yersinia* (Pruß *et al.*, 1997; Young *et al.*, 1999a). In addition, the *flhDC* mutation in *Xenorhabdus* was found to affect both haemolysis and lipolysis activity (Givaudan and Lanois, 2000). Therefore solid conclusions about the role of motility alone could not be drawn from these experiments. It was consequently decided to undertake a study into the role of motility in the life cycle of *Photorhabdus luminescens* TT01

The mutant BMM316 had an insert in the gene *flgG*, which is part of the *flgBJ* operon, and in *E. coli* and *Salmonella* this is a class II flagellar operon expressed midway through the regulatory cascade. The protein encoded by *flgG* forms part of the flagellar basal body and as such it is unlikely to control the expression of any other genes. The mutant BMM316 was therefore used to investigate the role of motility in the life cycle of a primary phenotypic variant of *Photorhabdus luminescens* TT01.

4.3.1 The Flagella Export Apparatus of BMM316

In *Yersinia enterocolitica* there are 5 extracellular proteins secreted by the flagella export apparatus that have no known function in motility (Young *et al.*, 1999a). One of these proteins is the phospholipase YplA required for host

pathogenicity and is also part of the flagella regulon (Schmiel *et al.*, 2000). The export and activity of YplA was abolished when a mutation was introduced into *flhDC*; however the activity was also abolished when a mutation was introduced into *flgF* (Young *et al.*, 1999a). In *Yersinia enterocolitica flgF* is in an operon of 9 genes that encode subunits of the flagella basal body indicating that an intact basal body structure is needed for secretion of phospholipase. The gene *flgG* is directly downstream of *flgF*, which raises the possibility that BMM316 also does not have a functional basal body and therefore cannot secrete proteins via the flagella export apparatus. However, it is known that lipase encoded by the gene *lip*-1 in *Photorhabdus temperata* K122 is not secreted by the flagella basal body in *Photorhabdus* and therefore a defect in lipase activity would not be expected in BMM316 (Wang and Dowds, 1993). The lipase defect apparent in BMM307 and the *Xenorhabdus flhD* mutant therefore suggests that the gene(s) involved is (are) under the regulatory control of FlhD.

In *E. coli* both FlgF and FlgG are located in the periplasmic space with FlgF forming part of the proximal rod and FlgG forming the distal rod. Interestingly neither appears to be necessary for the completion of the basic flagella export apparatus and are themselves exported by this mechanism before being assembled (Macnab, 2003). It is unclear which substructure is needed for export of non-flagella associated proteins. In *Yersinia* it is apparent that an incomplete basal body inhibits export (Young *et al.*, 1999a). However in *E. coli*, each stage of flagella construction after the basic export machinery involves a capping structure at the tip of the central export channel and each protein is likely to be distally incorporated under this structure (Macnab, 2003). It is therefore difficult to envisage how flagella structures assembled after the export machinery could allow the export of non-flagella related proteins as the export channel is effectively blocked by these capping proteins. Further characterisation of all secreted products would reveal whether BMM316 is affected in secretion of proteins through the flagella export apparatus.

4.3.2 BMM316 and Phenotypic Variation

Motility and flagella production have been correlated with phenotypic variation. The primary phenotypic variant can swim and swarm under appropriate

conditions whereas the secondary cannot and, in *Xenorhabdus*, the secondary variant does not appear to produce flagellin (Givaudan *et al.*, 1995). However, recent data have suggested that *Photorhabdus* secondary variants can be motile under appropriate anaerobic conditions whilst maintaining the majority of the other characteristics representative of secondary phenotype (Hodgson *et al.*, 2003). This indicates the regulatory system controlling the secondary characteristics in *Photorhabdus* can be divided and motility can be controlled by oxygen levels in a manner independent of the other characteristics (Hodgson *et al.*, 2003) Moreover, the suppression of many of the primary characteristics in the secondary variant has been shown to be controlled by *hexA*; however motility was not affected in a *hexA* mutant, further indicating motility is separately controlled (Joyce and Clarke, 2003).

4.3.3 BMM316 and Pathogenicity

Silva *et al* (2002) performed studies on the pathogenicity of the insect host *Manduca sexta* by *Photorhabdus luminescens* subspecies *akhurstii* W14 and found a distinct pattern of infection. *Photorhabdus* cells multiplied in the midgut region and haemolymph following injection. The colonisation of the gut tissue first occurred at the anterior region then progressed posteriorly suggesting a specific programme of infection by *Photorhabdus*. Moreover the characteristic expansion of the infection area suggested a role for bacterial motility *in vivo*.

However, the non-motile aflagellate mutant BMM316 was as virulent as the parent strain as shown by LT_{50} and LD_{50} towards *G. mellonella*. Further, when placed in direct competition inside the insect larvae BMM316 is able to colonise and multiply to approximately the same number as the competing motile *Photorhabdus* cells (Fig. 4.7). Interestingly these results clearly indicate for the first time flagella mediated motility does not have a significant role in the pathogenicity of *Photorhabdus luminescens* TT01 towards *G. mellonella*. However, it is unknown if the characteristic spread of infection as seen in *M. sexta* is also present in *G. mellonella*. Further, it is unknown if this putative motility is flagella based, such as swimming or swarming, or pili based, such as twitching. Lastly the virulence of *Photorhabdus* for insect hosts depends on the strain of *Photorhabdus* and insect host used, as well as age and physiological

state of the insect (Akhurst and Dunphy, 1993). Therefore the mechanism of pathogenicity of *Photorhabdus* may vary, e.g. *Photorhabdus luminescens* subspecies *akhurstii* W14 is orally toxic, whereas *Photorhabdus luminescens* subspecies *laumondii* TT01 is not (Marokhazi *et al.*, 2003). Therefore it is difficult to draw conclusions from experiments using other systems, i.e. *M. sexta* and W14, and the mechanism of pathogenicity may need to be determined for each *Photorhabdus*-insect interaction.

Lipase and haemolysin are assumed to be important during the insect associated stage of the life cycle of *Photorhabdus* and *Xenorhabdus* when the insect tissue is being broken down. Further to this Rosner *et al.*, (1996) found very low levels of oxygen just below the cuticle of a dead insect and effective anaerobis deeper in the cadaver. Under these conditions the secondary variant of *Photorhabdus* would be expected to be motile (Hodgson *et al.*, 2003) and secrete lipase and haemolysin, through the action of *flhDC* on the expression of the respective genes, further suggesting a role for motility and these products in the insect host.

4.3.4 BMM316 and Symbiosis

Prior to this work, the effect of motility on the symbiosis of *Photorhabdus* with *Heterorhabditis* had not been explored. Experiments with BMM316 showed flagella mediated motility is not essential for growth and development of the nematode as both TT01 and BMM316 allowed the full nematode developmental cycle to occur, and nematode yield is approximately the same for both strains, *in vivo* and *in vitro* (Fig. 4.3 and data not shown).

Photorhabdus are found in the intestine of the IJ stage of their respective nematode hosts (Ciche and Ensign, 2003). After growth and reproduction of *Heterorhabditis bacteriophora* on *Photorhabdus luminescens* TT01, each emerging IJ nematode contained approximately 160 cells. However each emerging nematode grown on BMM316 contained, on average, 25 cells. This lower level of colonisation, although significant, does not affect the ability of these nematode/bacterial complexes to subsequently infect and kill *G. mellonella* larvae. This indicates that the nematode normally retains many more bacteria than it needs to and, importantly, motility is not essential for the release of

Photorhabdus cells from the nematode upon penetration into the insect. Therefore, flagella mediated motility is not essential for either the colonisation of the nematode partner or the completion of the life cycle. However flagella do have a significant role in the final number of cells found in each emerging IJ, although the exact role that flagella have in colonisation has yet to be elucidated. Flagella may be important in the initial recognition of *Photorhabdus* by the nematode or they may be important in translocation inside the nematode to a specific niche. The mutant BMM316 is non-motile due to the absence of flagella and in order to fully explore the role of motility in the life cycle of TT01 a normally flagellate but non-motile mutant should be made and tested to assess which is actually required for colonisation, motility or flagella.

In the *Xenorhabdus nematophila/Steinernema carpocapsae* symbiosis recent data suggests that one or two *Xenorhabdus* cells colonise the nematode and these cells grow inside the specialised sac to form the final population (Martens *et al.*, 2002). There is evidence to suggest there may be a similar mechanism in the *Photorhabdus luminescens* and *Heterorhabditis bacteriophora* symbiosis and that these bacteria form a biofilm like structure inside the intestine (R. Watson, unpublished data; ffrench-Constant *et al.*, 2003). Earlier work with *Heterorhabditis* indicated that *Photorhabdus* cells could be found throughout the lumen of the nematode intestine (Endo and Nickle, 1991); however recent work by Ciche and Ensign (2003) has suggested a more defined area for colonisation; the anterior midgut. Furthermore TEM of the intestinal region of *Heterorhabditis* clearly shows close interactions between *Photorhabdus* cells, both with each other and with the nematode gut epithelium (ffrench-Constant *et al.*, 2003) suggesting a specific physical interaction between symbiont and host, and not simply a planktonic presence in the lumen.

The close bacterial-host interactions observed are likely to be mediated by physical appendages on the bacterial cell surface such as flagella, pili, fimbrae, membrane proteins or LPS. Bacterial cell surface structures have been well documented as having roles in bacteria-host interactions. In addition flagella have been implicated as being important in initialising attachment during biofilm formation (for a review see O'Toole *et al.*, 2000; Lawrence *et al.*, 1987).

Moreover type IV pili are essential for twitching and this has been shown to be important for microcolony formation during biofilm development (O'Toole and Kolter, 1998). Furthermore LPS also has roles in biofilm development and correct LPS structure has been shown to be necessary for the symbiosis of *Sinorhizobium* with alfalfa (O'Toole *et al.*, 2000; Campbell *et al.*, 2002). Therefore these physical appendages represent an interesting target for research into the interactions of *Photorhabdus* and *Xenorhabdus* with their respective nematode hosts.

Flagella and motility are often important in biofilm formation, although not always essential under specific environmental conditions (Klausen *et al.*, 2003). Furthermore *in vitro* biofilm architecture is highly dependent on the type of growth media and surface substrate indicating a complex set of environmental signals and bacterial responses are necessary for a range of biofilm developmental pathways (Klausen *et al.*, 2003). Therefore in the above model of nematode colonisation BMM316 would still locate to the intestine by nematode ingestion, but may form a biofilm with altered architecture. Alternatively, BMM316 may form a slower growing biofilm as it does *in vitro* resulting in a lower number of *Photorhabdus* cells in the mature IJ.

When in direct competition with TT01 for the colonisation of IJs the level of BMM316 colonisation falls and the median ratio of BMM316 to TT01 present was 0.0368. The manufacture of flagella and the function of chemotactic motility are energetically expensive and it has been suggested that a non-motile mutant strain of *E. coli* would have a 2% growth rate advantage over flagellated cells (Macnab, 1992). Therefore, BMM316 should have a slight growth advantage over TT01. Indeed on lipid agar plates after three weeks incubation BMM316 is present in higher numbers than TT01. However due to the very low ratio of BMM316 to TT01 CFUs present when in competition inside the nematode it can be concluded that flagella offer a significant advantage during colonisation. In the above model, based on growth of a few cells in a possible biofilm, the motile cells would out compete the non-motile cells in initiating, growing and maturing in a biofilm, leading to a significantly lower number of non-motile cells in the mature IJ. Interestingly, there appears to be several rounds of growth and

selection in the nematode. Recent data shows a steady overall increase in numbers of *Photorhabdus* CFUs present inside *Heterorhabditis* IJs over time, but with periods of growth and decline (R. Watson, personal communication). This data supports previous data by Martens *et al.*, (2003) who found the same phenomenon with *Xenorhabdus* and *Steinernema*. If TT01 were able to out-compete BMM316 during biofilm formation this would be exacerbated over the several rounds of growth and decline.

IJs propagated on the *Photorhabdus* parent strain do not all contain the bacterial symbiont and approximately 5% of these IJs are axenic (Ciche and Ensign, 2002). Interestingly nematodes grown on *Photorhabdus* containing a GFP construct randomly inserted into the chromosome have a higher axenic population of 74% (Ciche and Ensign, 2002). Another model would therefore suggest that individual nematodes propagated on BMM316 contain a similar number of bacterial cells as those propagated on TT01; however a higher proportion of the IJ population would be axenic. Ultimately a combination of the growth-based and colonisation-based models is possible. The correct colonisation of *Steinernema* and *Heterorhabditis* IJs and growth by their bacterial symbionts is likely to be a complex and highly regulated event. This event involves many different genes including genes encoding a structural protein (FlgG) (this study), an enzyme (NgrA) (Ciche *et al.*, 2001) and a regulatory protein (σ^S) (Vivas and Goodrich-Blair, 2002). In conclusion, during colonisation, motility or flagella provide a significant advantage to *Photorhabdus*. Although flagella are not involved in pathogenicity of the insect host the selective pressure during symbiosis and colonisation would mean *Photorhabdus* retains the genes required for flagella production and function.

CHAPTER 5

5.0 The *pbgPE* Operon of *Photorhabdus* is Required for Pathogenicity of *Galleria* and Colonisation of *Heterorhabditis*.

5.1 Introduction

Bacterial motility is a highly coordinated activity. As shown in previous chapters numerous mutations, without obvious links to flagella synthesis, can affect the ability of a bacterium to swim. Defects in the normal composition or functioning of a membrane can affect a bacterial cell in a number of ways including its motility through semi-solid media. *Photorhabdus* are Gram-negative, that is they possess an outer membrane primarily consisting of phospholipids and lipopolysaccharides (LPS) (See Chapter 1, Fig 1.2). Most bacteria also have a characteristic composition of proteins in this outer membrane, which can be detected as a recognisable pattern of bands on a protein gel. They include lipoproteins, porins and other proteins involved in diffusion processes. The inner membrane acts as a barrier with import and export channels as the entry of many molecules require specific proteins for their uptake (Cronan *et al.*, 1987).

LPS comprises the majority of the outer leaflet of the outer membrane. LPS itself is composed of three segments including a lipid A segment that is negatively charged through the possession of phosphate groups. Lipid A is also hydrophobic through the presence of acyl chains that anchor the LPS in the outer leaflet and form part of the lipid bilayer. The core is the next segment and is comprised of sugar units typically containing heptose. The last segment is the O antigen, which protrudes from the lipid bilayer and is hydrophilic. The O antigen is comprised of varied, repeating sugar units.

Flagella are anchored in the bacterial cell wall by the basal body, which consists of a number of proteins including a protein ring (MS ring) (encoded by *fliF*) localised to the inner membrane, the P protein ring (encoded by *flgI*) localised to the peptidoglycan layer of the bacterial cell wall and then the L protein ring

(encoded by *flgH*) localised to the outer membrane. Thus the flagellum forms interactions with components of the inner and outer membranes and the peptidoglycan layer of the bacterial cell wall.

Mutations in proteins that either contribute to the structure of a bacterial membrane or form part of the membrane can be hypothesised to affect motility in two ways. First, changes in the inner or outer membrane structure or composition may alter the interactions of the flagella basal body with the respective membrane layer leading to alterations in normal flagella function. For example, the switch proteins involved in the switching of rotation of flagella are peripherally mounted onto the MS ring in the cytoplasmic membrane (Macnab, 1996). Disruptions in the cytoplasmic membrane that alter the activity of this protein could result in unidirectional rotating flagella with either CW or CCW bias. Even with a bias towards straight swimming mutants showed slow spreading in swim agar (Macnab, 1987). Second, alterations in membrane structure or proteins may alter the movement of nutrients and waste across the membrane affecting the growth of the cell. This defect in growth could cause the affected bacteria to spread slowly through semisolid media and giving the appearance of being affected in motility. The mutant BMM305 was originally identified in the screen for mutants defective in motility. The transposon was identified as being in *pbgE1* a gene with homology to *pmrK* in *Salmonella enterica* (See Chapter 3, Table 3.1). In *S. enterica* a mutation in *pmrK* resulted in changes in LPS and an increased sensitivity to antimicrobial peptides; however the authors did not test for motility defects (Baker *et al.*, 1999). Homology of the surrounding genes in *Photorhabdus* to *Salmonella* suggests a similar role for *pbgE1* in TT01. This chapter investigates the function of *pbgE1* in the production of LPS and the role this may have in the life cycle of *Photorhabdus*.

5.2. Results

5.2.1 Identification of Insertion Site and Complementation

The transposon insertion site in BMM305 was identified as being in nucleotide 1281 of *pbgE1*, a 1662bp gene with 45% identity, at the amino acid level, to

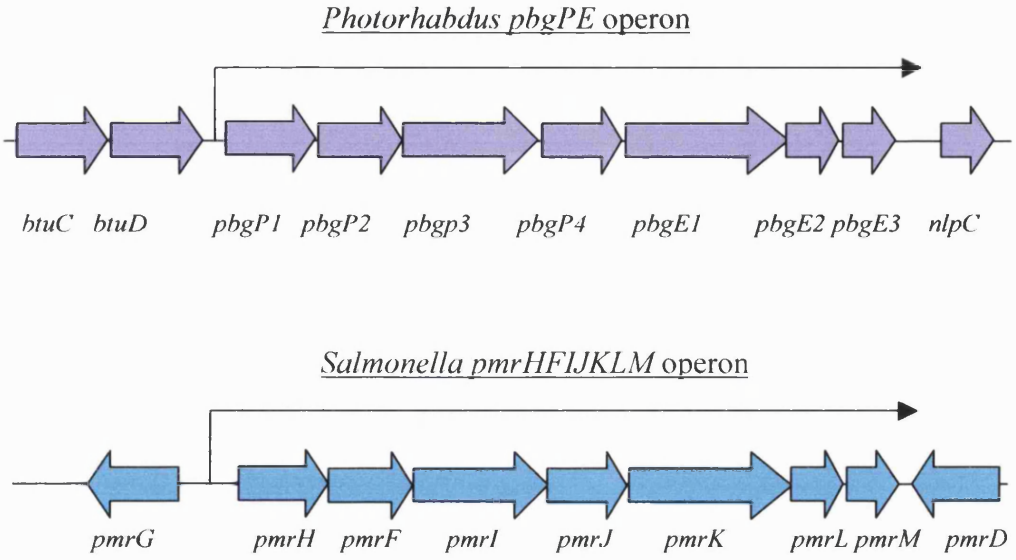


Figure 5.0. Comparison of the *Photorhabdus pbgPE* operon, the *Salmonella pmrHFIJKLM* operon and surrounding genes.

pmrK in *Salmonella*. In *Salmonella* the gene *pmrK* is part of a seven-gene operon comprising *pmrHFIJKLM* (Fig. 5.0). This operon encodes biosynthetic enzymes for the production and addition of either one or two 4-amino-4deoxy-L-arabinose (L-Ara4N) molecules to the lipid A moiety of lipopolysaccharide (Trent *et al.*, 2001). *In silico* analysis of the TT01 genome reveals that TT01 appears to share the organisation of this operon with the *pbgP1P2P3P4E1E2E3* genes being 63, 69, 68, 59, 45, 46 and 43% identical, at the amino acid level, to the respective genes in *Salmonella*. This homology suggests that the genes may have a similar role in membrane modification in TT01. However it is interesting to note that the two operons do not appear to be at the same location in the genome as the genes surrounding the operons differ. In *Salmonella* the operon has *pmrD* downstream and *pmrG* upstream. These genes are involved in the regulation of the *pmrHFIJKLM* operon (Gunn *et al.*, 2000). In *Photorhabdus* the genes upstream of the *pbgPE* operon have homology to the *btuCD* genes, which are involved in vitamin B_{12} transport. The predicted protein product of the gene downstream gene has homology to the lipoprotein precursor NlpC. Furthermore, in

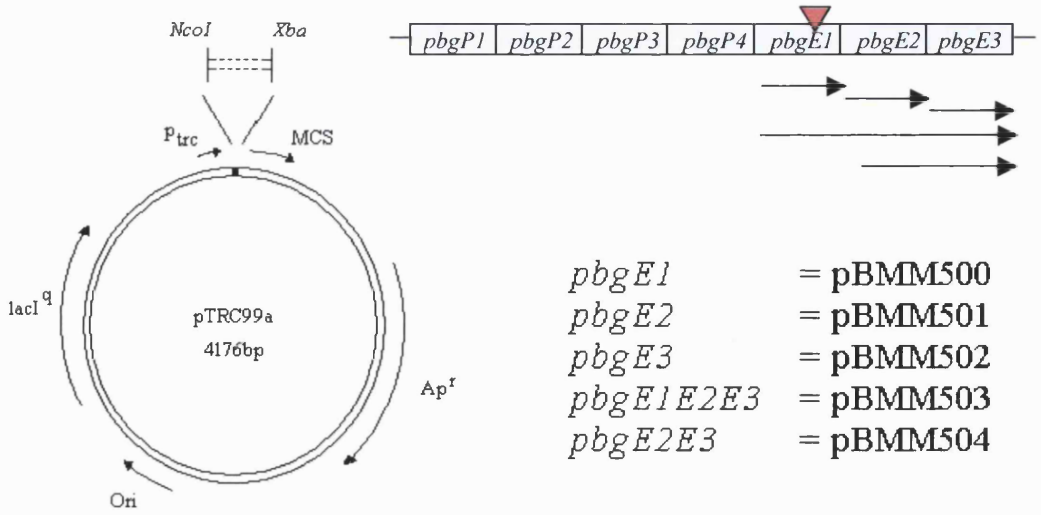


Figure 5.1. Genes cloned into *NcoI* and *Xba* restriction sites, in the multiple cloning site (MCS) of pTRC99a and resulting plasmids. Red arrow indicates site of transposon insertion in BMM305.

Salmonella the two-component pathway PmrAB regulates the expression of *pmr* genes (Gunn *et al.*, 1998). *In silico* analysis revealed that *P. luminescens* lacks this two-component pathway suggesting a different method of regulation (Derzelle *et al.*, 2004a).

5.2.2 BMM305 is Impaired in Motility

BMM305 was isolated as being defective in motility; it is approximately 22% less motile than TT01 after 48h incubation at 28°C. In order to determine whether the phenotypes observed in BMM305 are due to the interruption of *pbgE1* or downstream polar effects on the rest of the putative operon, BMM305 was complemented with combinations of downstream genes cloned into the pTRC99a plasmid, as described in Materials and Methods. These include *pbgE1*, *pbgE2*, *pbgE3*, *pbgE1E2E3* and *pbgE2E3,* therefore constructing plasmids pBMM500, pBMM501, pBMM502, pBMM503 and pBMM504, respectively (Fig. 5.1). These plasmids were electroporated into TT01 and BMM305 as a control and tested for their effect on motility (5.2).

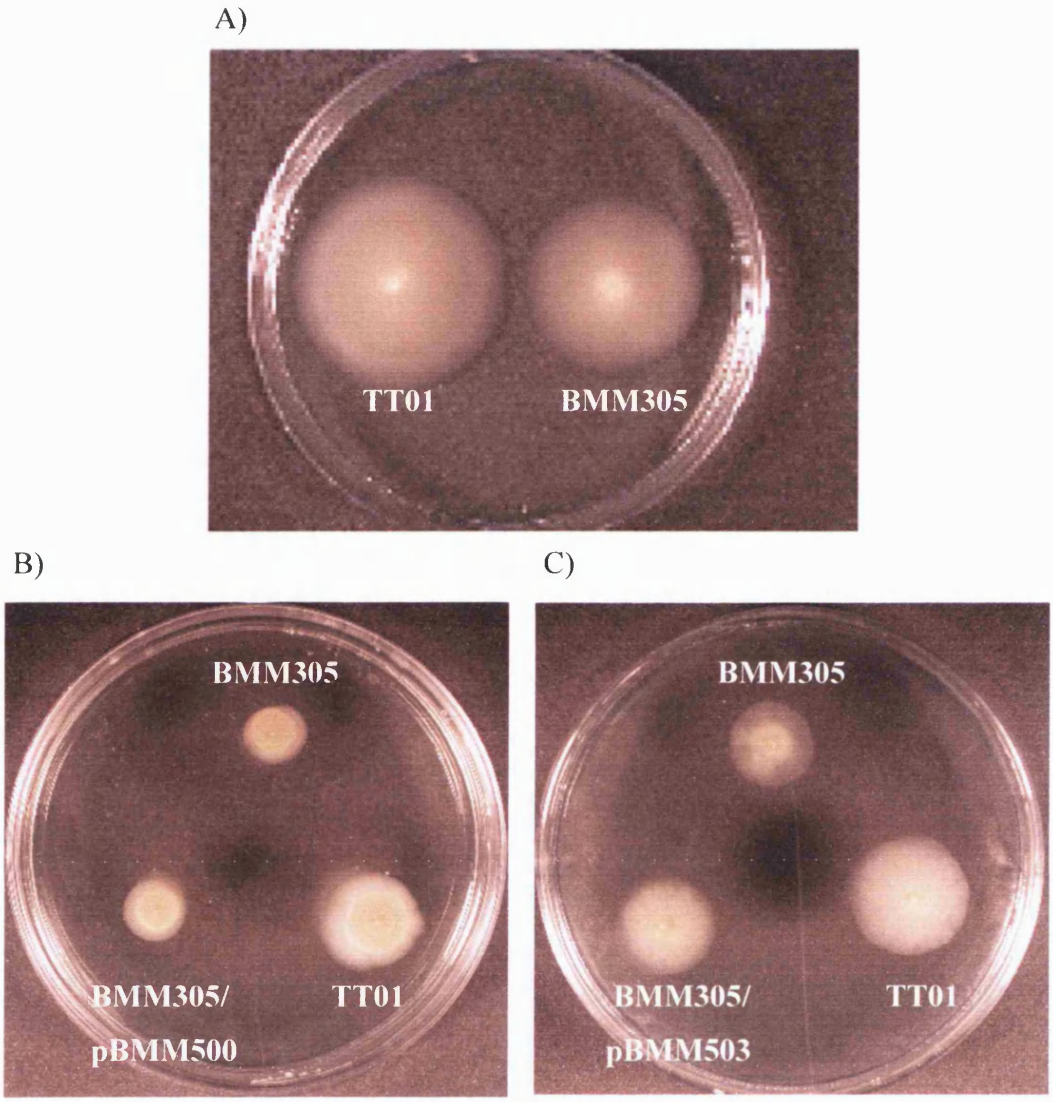


Figure 5.2. 0.3% Swim agar plate inoculated with 3µl of overnight culture resuspended to an OD_{600} 1.0, from A) *Photorhabdus luminescens* TT01 and BMM305, incubated at 28°C for 48h. B) *Photorhabdus luminescens* TT01, BMM305 and BMM305 /pBMM500 (*pbgE1*), incubated at 28°C for 24h. C) *Photorhabdus luminescens* TT01, BMM305 and BMM305/pBMM503 (*pbgE1E2E3*), incubated at 28°C for 24h.

Phenotype		TT01	BMM305
Dye absorption	Mc Conkey	+	+
	EMB	+	+
	NBTA	+	+
Bioluminescence		+	+
Catalase		+	+
Crystalline inclusion proteins		+	+
Extracellular products	Lipase	+	+
	Protease	+	+
	Antibiotics	+	+
	Siderophores	+	+
Motility		+	W+
Biofilm formation		+	+
Colony morphology on LB		Round, mucoid	Round, mucoid
Pigmentation on LB		Yellow/brown	Yellow/brown

Table 5.0. Phenotypic tests and the results for wild type *Photorhabdus luminescens* TT01 and BMM305. + indicates a positive result, W+ indicates a weakly positive result.

BMM305/pBMM500 failed to restore full motility (Fig. 5.2 B). However BMM305/pBMM503 did restore motility to parental levels (Fig. 5.2 C) indicating that the full operon is needed to restore the ability to swim and therefore the insertion may be affecting the downstream genes.

5.2.3 BMM305 is Primary-Like in All Other Characteristics

To assess whether the insertion in BMM305 had any other effects on the standard primary characteristics of TT01 phenotypic tests were carried out on BMM305 including dye uptake, exoenzyme production and bioluminescence (Table 5.0). BMM305 was shown to be primary like in all characteristics tested and only differed from TT01 in motility, where it was 22% less motile.

5.2.4 BMM305 is Slightly Impaired in Overnight Growth Yield

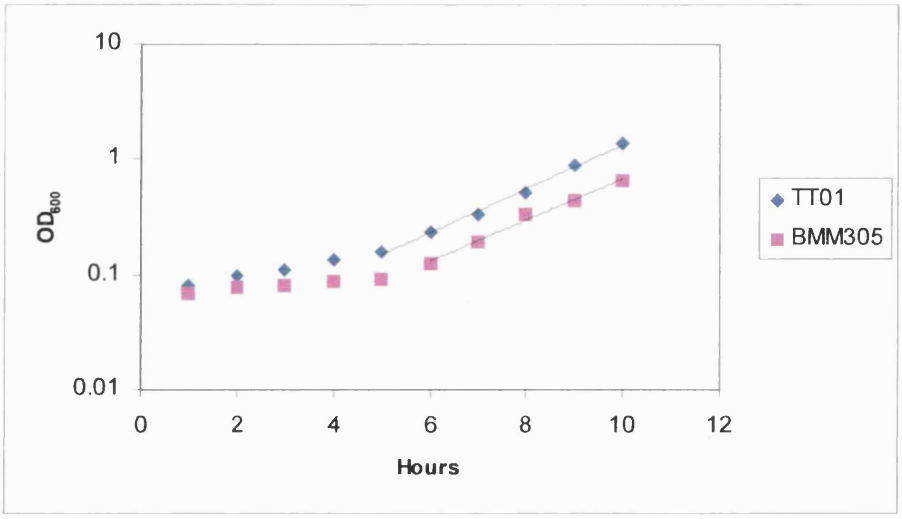
Bacteria that harbour mutations in membrane proteins or genes that contribute towards a normal functioning membrane can be affected in normal growth as discussed in Chapter 3. As BMM305 has an insertion in a region with strong homology to genes involved in lipid A modifications a putative defect in membrane function or composition has been hypothesised. In order to assess what affect this putative membrane defect may have on the growth of the strain growth curves were carried out in triplicate (Fig. 5.3 A represents one repeat). The strains were inoculated from overnight cultures into conical flasks to the same OD_{600}, grown at 28°C at 200rpm and OD_{600} measurements were taken every hour. TT01 has a slightly shorter lag phase than BMM305 as TT01 reached exponential phase first after approximately 5h. During exponential growth TT01 had a doubling time (t_d) of 1.58h. BMM305 reached exponential phase after approximately 6h and had a slightly slower t_d of 1.68h. After overnight growth BMM305 reaches a lower OD_{600} (OD_{600} = 6.09) than TT01 (OD_{600} = 7.74) (data not shown); however BMM305 pigments and produces all the phenotypic characteristics associated with primary stationary phase growth.

The complemented strain BMM305/pBMM503 does not appear to have a lag phase defect (Fig. 5.3 B). Both strains were inoculated to the same OD_{600} and grew to the same extent in lag phase and both TT01 and BMM305 appear to enter exponential phase at the same time. This experiment was repeated three times and the data shown is one representative repeat, these results suggest that the *in trans* expression of *pbgE1E2E3* complemented the lag phase defect.

5.2.5 BMM305 Lacks an O Antigen

In *Salmonella*, the *pmrHFIJKLM* operon encodes genes involved in the production and addition of $_L$-Ara4N to the lipid A moiety of LPS (Gunn *et al.*, 2000). This addition is essential for the *in vivo* survival and virulence of *Salmonella* and a mutation in *pmrK* prevents this modification (Baker *et al.*, 1999). The homology of the gene products of the *pbgPE* operon to the products of the *pmrHFIJKLM* operon, suggest a role for *pbgPE* in LPS modification. LPS

A)



B)

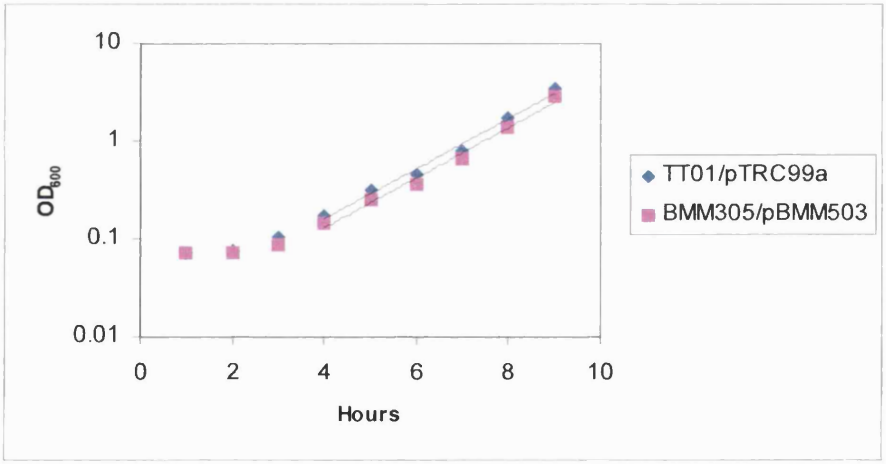


Figure 5.3. A) The growth (OD_{600}) of *Photorhabdus luminescens* TT01 and BMM305 and B) *Photorhabdus luminescens* TT01 and BMM305/pBMM503. The strains were inoculated from overnight cultures into conical flasks to approximately the same OD_{600}, grown at 28°C at 200rpm and measurements were taken every hour.

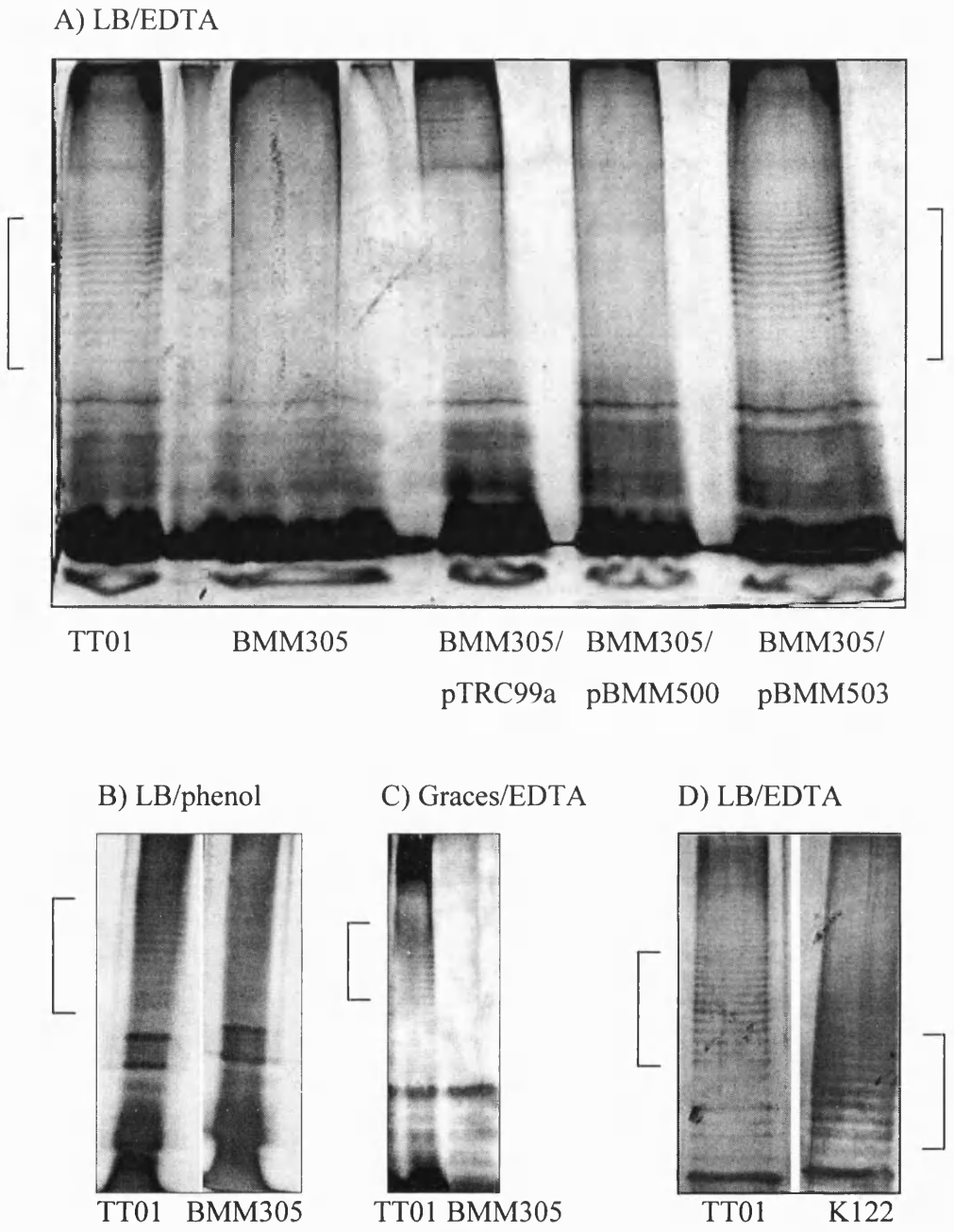


Figure 5.4. Silver stained SDS polyacrylamide gels of LPS from cultures grown in: A) LB and prepared with EDTA, B) LB prepared with phenol, C) Graces media prepared with EDTA and D) LB prepared with EDTA. A) LPS from strains grown in LB; TT01 and BMM305/pBMM503 show the presence of O antigen bands. BMM305, BMM305/pTRC99a and BMM305/pBMM500 show the absence of O antigen. B) LPS prepared using phenol from 5mls of overnight culture from TT01 and BMM305. C) LPS prepared from

TT01 and BMM305 grown in Graces media. D) LPS prepared using EDTA from TT01 and K122. Brackets highlight areas of heavy staining indicating modal length O antigen.

from TT01 and BMM305 were prepared using an EDTA based method and run on a 12.5% SDS-polyacrylamide gel and stained using a modified silver staining protocol (Fig. 5.4 A). LPS prepared from TT01 shows several collections of bands including a ladder like organisation in the middle of the gel. These bands represent the O antigen section of LPS, which is a repeating sugar unit of varying length and the different bands on the gel represent different length O antigens. The darker more defined bands in the middle of this ladder represent a modal length of O antigen. The bands at the bottom of the gel represent the lipid A section of the LPS. The LPS preparation from BMM305 contains lipid A but does not contain any O antigen. To determine whether this was due to absence of O antigen or an artefact of extraction due to the membrane changes of this mutant, LPS was also extracted using a phenol based method (Fig. 5.4 B) and the same result was seen. From this it was concluded that BMM305 contained lipid A but lacked the O antigen of LPS. BMM305/pBMM500 did not appear to produce an O antigen, whilst BMM305/pBMM503 did produce an O antigen and has an LPS profile similar to TT01 (Fig. 5.4 A). In conclusion these results suggest the mutation in BMM305 may be polar and that the whole operon is needed for correct O antigen production.

The growth medium LB broth contains complex undefined nutrients for the growth of a wide variety of micro-organisms. *Photorhabdus* is also able to grow in the haemolymph of an insect to very high numbers and this environment is likely to be very different to that encountered in LB broth. In order to establish whether the environment has an effect on LPS production, TT01 and BMM305 were grown overnight in Graces media and LPS was prepared using the EDTA based method (Fig. 5.4, C). It can be clearly seen that TT01 produced LPS containing an O antigen in Graces media and BMM305 did not. These results strongly suggest that TT01 would produce an O antigen *in vivo* and BMM305

would not; therefore suggesting any differences in *in vivo* behaviour in BMM305 might be due to the lack of O antigen.

LPS was also prepared from *Photorhabdus temperata* K122 to compare the O antigen profiles. Both samples were isolated at the same time and treated to the same conditions and run on the same gel (Fig. 5.4 D). Interestingly, the results indicate that K122 LPS has a much smaller modal length of O antigen than TT01.

5.2.6 BMM305 Supports Nematode Growth and Development

LPS is a structure that is anchored in the outer leaflet of the outer membrane; however the majority of a complete lipopolysaccharide molecule lies external to the cell and as such is likely to physically contact hosts and elicit responses. Incorrect LPS may lead to the bacterial cell not being recognised as either a pathogen or a symbiont leading to altered host interactions. The correct LPS structure has been shown to be important in the symbiosis of *Sinorhizobium meliloti* with alfalfa, and mutants of *Sinorhizobium* that lack the correct lipopolysaccharide core structure have been shown to fail in sustaining a chronic intracellular infection of symbiotic nodules with the plant root (Campbell *et al.*, 2002). In order to study the role LPS may play in the first stage of the symbiotic association of *Photorhabdus* with its nematode partner *Heterorhabditis* (i.e. the ability to support nematode growth and development), BMM305 and TT01 were grown on lipid agar plates for 48h at 28°C and approximately 20 surface sterile IJs were added to each plate. An approximately equal number of IJs recovered to hermaphrodites on both sets of plates (data not shown) and the hermaphrodites on the plates containing TT01 and BMM305 both laid eggs at the same time, which then developed into males and females. After incubation large numbers of IJs started to emerge and migrate from the plates containing TT01 and BMM305. No qualitative differences could be seen in these *in vitro* symbiosis assays suggesting that the first stage of symbiosis, the ability to support the growth and development of the nematode, does not require the presence of a correct O antigen.

The emerging IJs were collected, surface sterilised and allowed to naturally infect *G. mellonella* in order to assess the role that the O antigen of LPS may have in the growth and development of the nematode partner *in vivo*. Within 48h the insects infected with nematodes collected from plates containing TT01 died and became pigmented (data not shown). However the insects infected with the nematodes from the plates containing BMM305 did not die and no infection took place. The insects remained healthy and when left for up to a week after infection many of the insects started to pupate indicating a healthy developmental status (data not shown). These results implied an important role for an O antigen either in the pathogenicity of TT01 towards the insect host or in the second stage of symbiosis with the nematode, i.e. the ability to recolonise the nematode gut after growth and development.

5.2.7 BMM305 is not Retained by the Nematode

In order to determine the effect O antigen may have on the ability of TT01 to recolonise the nematode partner IJ nematodes were collected after *in vitro* growth and reproduction on BMM305 and TT01. Approximately 1,000 surface sterile nematodes were crushed and the suspension plated out onto LB media. Approximately 150 CFUs are retained by IJ nematodes that have been cultivated on TT01; however, BMM305 is severely attenuated in colonisation of the nematode gut with approximately 1 cell present per nematode (Fig. 5.5). These results clearly suggest an essential role for O antigen in the colonisation of the nematode gut.

To determine whether the general presence of *Photorhabdus* O antigen is recognised by the nematode, or whether each cell must require the correct LPS, BMM305 was grown on lipid agar plates in a 1:1 ratio with TT01. Surface sterile IJs were seeded onto these plates and incubated at 25°C for 3 weeks. The IJs that emerged at the end of the three weeks were collected, surface sterilised and crushed to reveal the ratio present in the nematode gut. The median ratio of bacteria present (BMM305/TT01) on the lipid agar plate at time of IJ formation was established as being 0.72 (Fig. 5.6 A), which indicates slightly more TT01

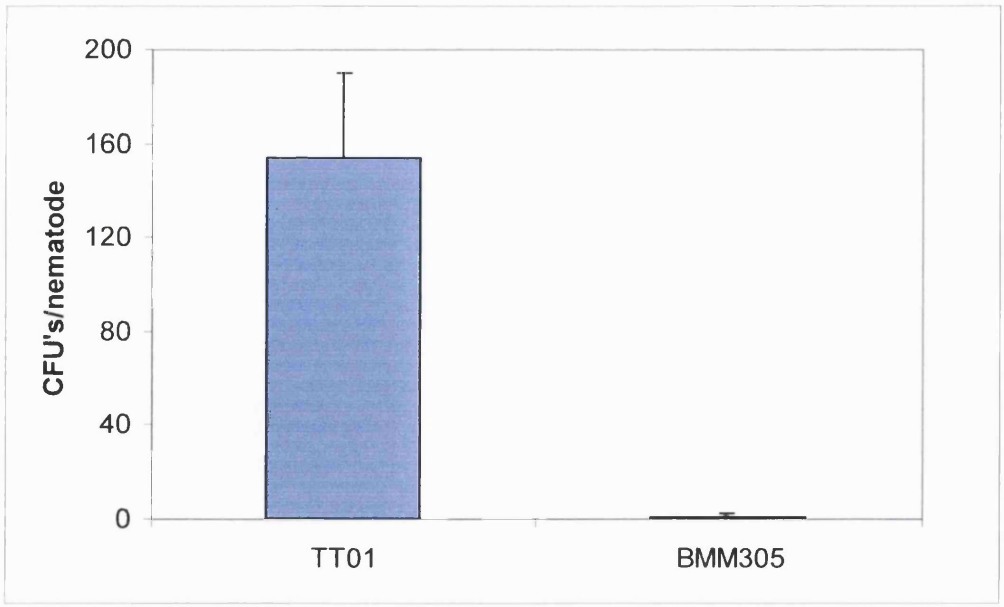


Figure 5.5. Average number of colony forming units (CFUs) of *Photorhabdus luminescens* TT01 and BMM305, obtained per nematode after growth and reproduction on each respective bacterial strain. Number of repeats as follows: TT01 = 20, BMM305 = 11.Error bars represent standard deviation.

were present than BMM305. However the median CI for BMM305/TT01 present in the IJ was less than 0.01 (the minimum value detected in this assay) (Fig. 5.6 B) suggesting that either the nematode fails to recognise BMM305 cells individually, or the cells themselves lack the ability to colonise the nematode as the lack of O antigen of BMM305 is not rescued by the presence of TT01. Unfortunately, experiments to study the complementation of this phenotype were unsuccessful. Strains of BMM305 carrying plasmids with *pbgPE* genes were grown on lipid agar plates and these were incubated with *Heterorhabditis* at 25°C for up to three weeks. At this time, when IJs were emerging, the bacteria were tested and found not to contain the plasmid. As growth and development of the nematode partner was unaffected by the mutation (Section 5.2.6) there was no selection pressure to maintain the plasmid. Therefore the second stage of this complemented symbiosis study, the recolonisation of the nematode host, could not be tested.

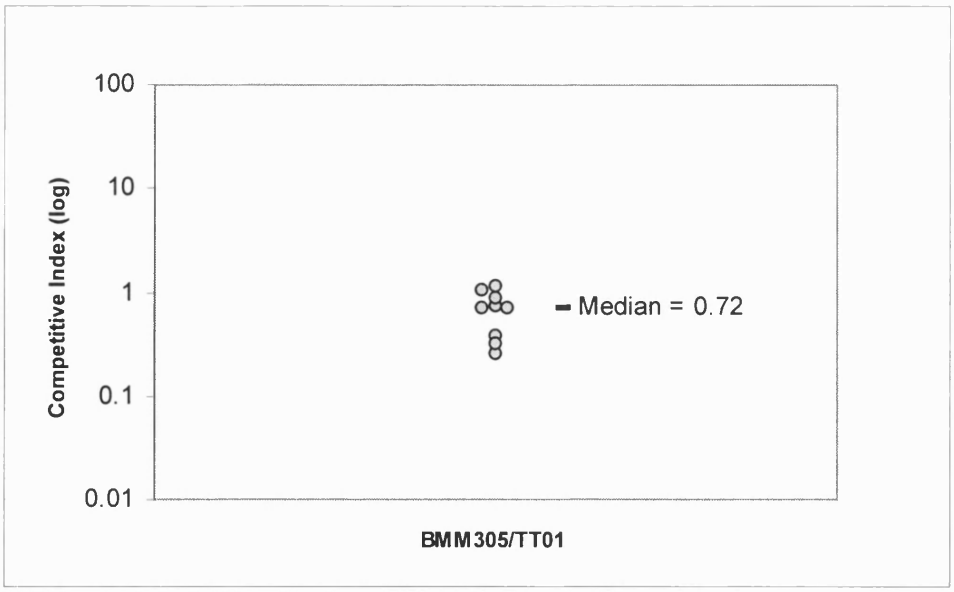


Figure 5.6 A *In vitro* competitive index (C.I.) of BMM305/TT01. Each point represents the ratio of bacteria harvested from individual plates at time of IJ formation (n = 9). The median C.I. for the 9 plates was 0.72.

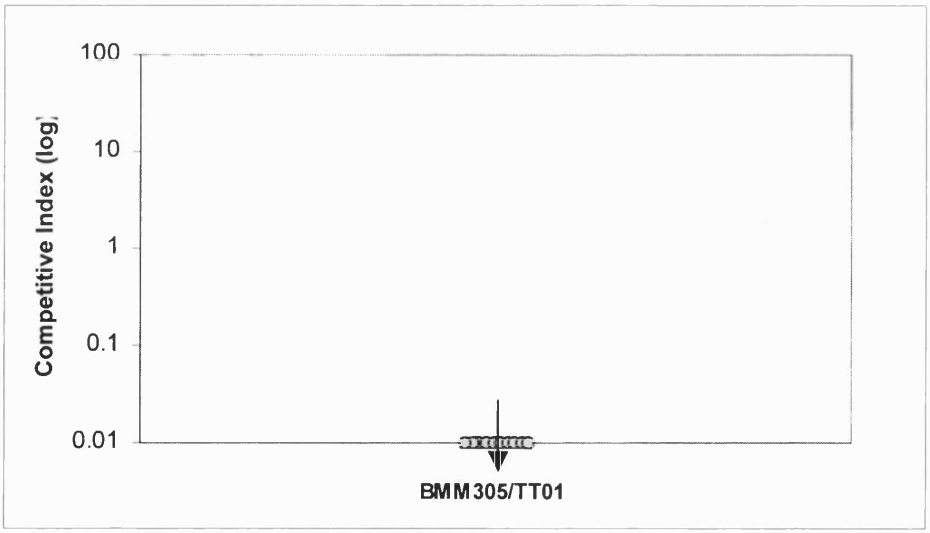


Figure 5.6 B *In vivo* symbiosis competitive index (C.I.) of BMM305/TT01. Each point (n = 9) represents the ratio of bacteria harvested from nematodes, from individual LA plates, after growth and reproduction on an equal mix of BMM305/TT01.

5.2.8 BMM305 is Attenuated in Virulence

Photorhabdus O antigen has been shown to be important in the correct association with its nematode partner *Heterorhabditis* (Fig. 5.5). Furthermore, correct LPS structure has been shown to be important in pathogenicity of hosts in other bacteria-host interactions (Murray *et al.*, 2003). To elucidate the role O antigen may play in the pathogenic interaction of *Photorhabdus* with its insect host 100 CFUs of both TT01 and BMM305 were injected separately into *G. mellonella*. Within 48h 100% of insects injected with TT01 were dead, however none of the insects injected with BMM305 died. The insects were then left at 25°C for a further 48h and at this time all the insects were still alive. A LD_{50} test (Fig. 5.7) showed that BMM305 does not begin to kill *G. mellonella* until at least 5000 CFUs are injected per insect and at this dose it takes 96h for approximately 6% of insects injected to die. We determined that the LD_{50} for BMM305 was approximately 1.02×10^5 CFUs per insect at 96h incubation at 25°C. The time of 96h incubation was chosen because at all doses of CFUs injected the insects did not start to die until between 48-72h post infection; however by 96h all sick insects died. Approximately 10^6 CFUs need to be injected per insect to ensure that larval death reaches approximately 97% after 96h. Taken together these results suggest an important role for O antigen in virulence as BMM305 is highly attenuated needing 5 orders of magnitude more CFUs to be injected and twice the length of time of TT01 to achieve >95% larval death. A quantitative LT_{50} was difficult to perform due to the high CFUs needed to achieve >95% death. Therefore LPS itself may be important in insect virulence or LPS may contribute towards survival within the insect environment.

It has been suggested that LPS may act to overcome certain aspects of the hosts immune response (Dunphy, 1995; Dunphy and Hurlbert, 1995), therefore LPS may work when supplied *in trans* in combination with BMM305. In order to further understand the role of LPS in the pathogenicity of *Photorhabdus* towards its insect host 100 CFUs of a 1:1 ratio of TT01 to BMM305 cells were injected into *Galleria*. The insects were incubated at 25°C and all died within 48h. Upon death the insects were surface sterilised, dissected in LB and plated out onto

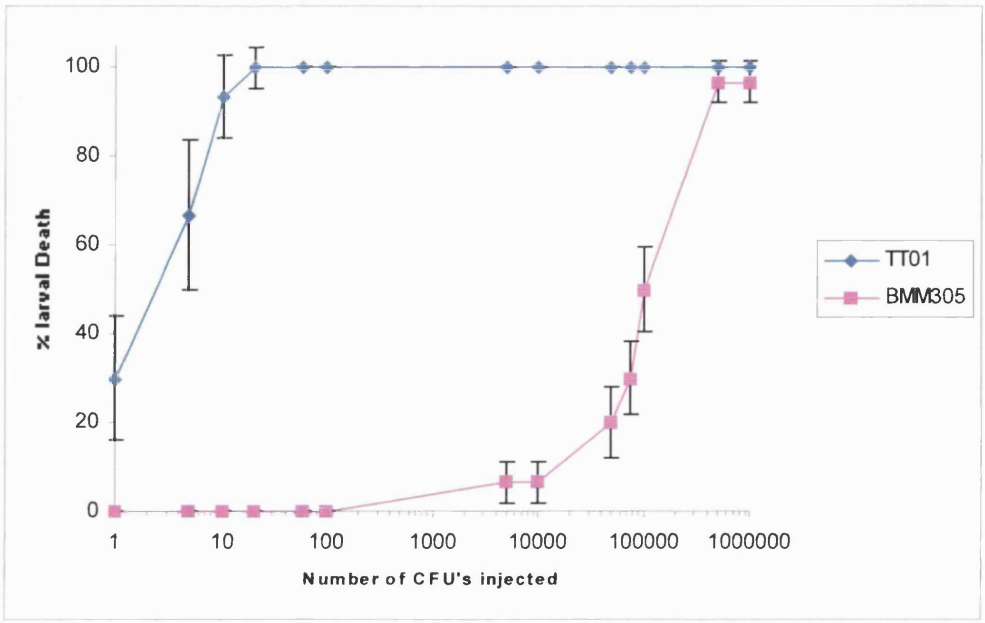


Figure 5.7. LD_{50} of *Photorhabdus luminescens* TT01 and BMM305 at 96h post injection. Different amounts of CFUs were injected and each dose was repeated in triplicate. For TT01, the LD_{50} was calculated as <5 CFUs per insect and 100% larval death is reached by injection of 20 CFUs per insect. For BMM305 the LD_{50} was calculated as 102,000 CFUs per insect and approximately 95% larval death is reached by injection of 500,000 CFUs. Error bars represent standard deviation over three repeats.

selective media in order to elucidate the ratio present at the time of death. Remarkably, 100% of *Photorhabdus* cells recovered from the dissected insects were TT01 (data not shown). BMM305, which was present in equal numbers at t_0, had not been able to grow in the insect environment even with LPS bearing TT01 cells present to overcome the immune response and kill the insect. These results suggest the O antigen is a specific characteristic that contributes towards cell survival within the insect.

5.2.9 Complementation of the Attenuated Virulence Phenotype

It has already been established that the *in* trans expression of *pbgE1E1E3* was sufficient to restore O antigen production in BMM305 (Fig. 5.4). In order to

determine what effect the presence of O antigen has on pathogenicity towards its insect host BMM305/pBMM500 (*pbgE1*) and BMM305/pBMM503 (*pbgE1E1E3*) were injected into *G. mellonella* at three different concentrations with TT01, BMM305 and BMM305/pTRC99a as controls. These strains were grown up overnight, the OD_{600} was measured and the cultures all resuspended in PBS to give injectable doses of 100, 1000 and 10000 CFUs. The injected *G. mellonella* larvae were incubated at 25°C and checked at 96h for % death. As expected the larva injected with TT01 at all three dilutions were all dead within 48h (Fig. 5.8). On the other hand only 13%, 10% and 20% of insects injected with 100, 1000 and 10,000 CFUs, respectively, of BMM305/pTRC99a died within 96h (Fig. 5.8). Moreover none of the insects injected with 100 CFUs of BMM305/pBMM500 died, whereas 6% and 20% injected with 1000 and 10000 CFUs respectively died within 96h. These numbers suggest that the *in trans* expression of *pbgE1* is not sufficient to restore the pathogenicity of BMM305. Interestingly injection of only 100 CFUs of BMM305/pBMM503 resulted in 96% insect death within 96h suggesting that *in trans* expression of *pbgE1E2E3* restores pathogenicity to BMM305. However it did not completely restore virulence as, although 100 CFUs resulted in >95% larval death, the time taken to reach this level was 96h, which is approximately twice that taken by TT01. A similar delay effect was seen with an avirulent *Photorhabdus phoP* mutant (Derzelle *et al.*, 2004a). The *in trans* expression of *phoP* restored dosage based virulence, but with a delay (Derzelle *et al.*, 2004a). This delay may be because of the added metabolic burden on the cell to replicate the plasmid. Alternatively, the amount and time of expression of the *pbgE1E2E3* genes on the plasmid may not be the same as parental expression of the *pbgPE* genes on the chromosome. In conclusion, the expression of *pbgE1E2E3* is needed for both O antigen production and pathogenicity in TT01, which suggests the correct O antigen is needed for survival and virulence within the insect host

5.2.10 BMM305 is Sensitive to Antimicrobial Peptides

In *Salmonella* a mutation in *pmrK* results in the cell being unable to modify the LPS with $_{L}$-Ara4N (Baker *et al.*, 1999; Gunn *et al.*, 2000). This molecule is

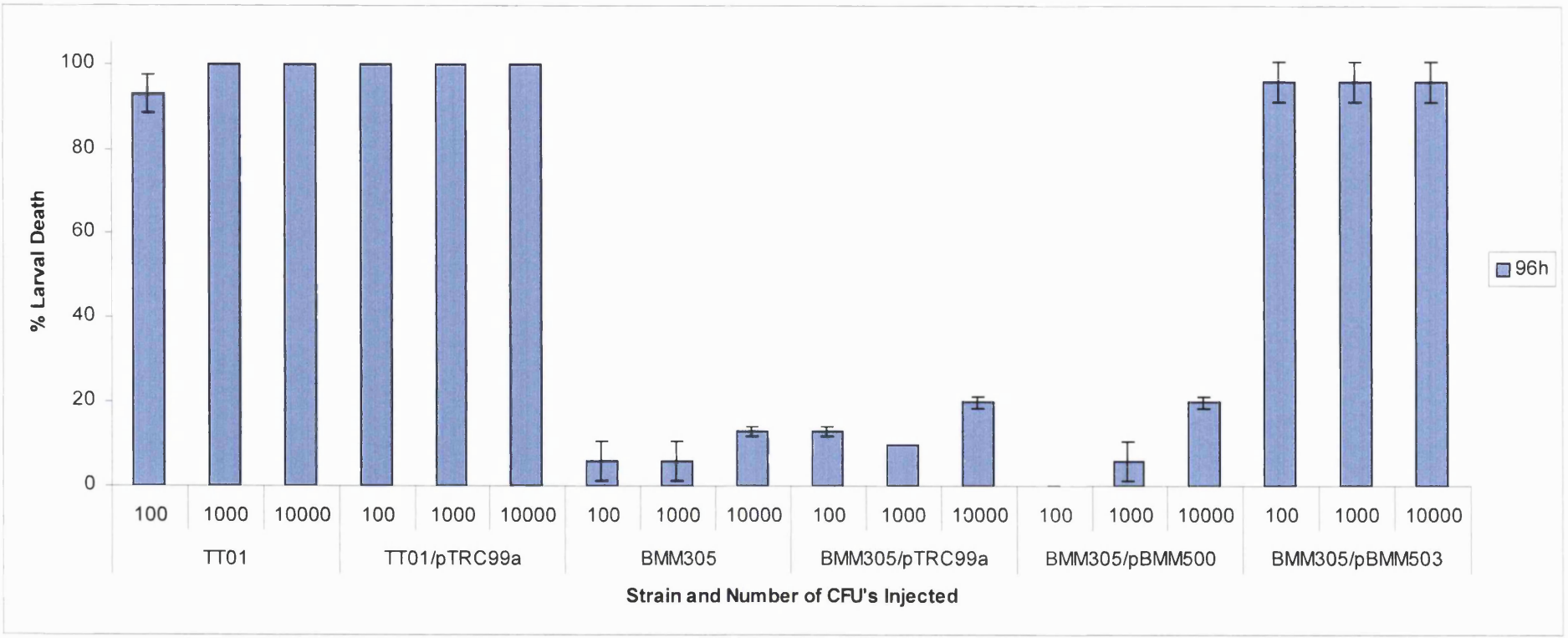


Figure 5.8. Pathogenicity of *Photorhabdus luminescens* TT01, TT01 pTRC99a, BMM305, BMM305 pTRC99a, BMM305 pBMM500 (*pbgE1*) and BMM305 pBMM503 (*pbgE1E2E3*) at 96h. Each strain was injected into *Galleria mellonella* at 3 different concentrations of CFUs, 100, 1000 and 10000. Bars represent an average of 30 insects. The results shown are an average of 3 independent experiments and Y error bars represent standard deviation.

positively charged and thus reduces the overall negative charge of LPS. Moreover, this change in charge will affect the cells interactions with other molecules in the environment. Antimicrobial peptides are small cationic amphipathic molecules that are bactericidal for most Gram-negative bacteria (Vaara, 1992). Polymyxin B is an antimicrobial peptide that binds the bacterial outer membrane through interactions with LPS. This binding makes the outer membrane permeable and allows polymyxin to have access to the inner membrane. The polymyxin can then permeabalise the inner membrane where it causes leakage of cellular contents and death (Vaara, 1992). Cells that can increase the positive charge on their membranes can decrease the attraction of positively charge molecules and are thus more resistant to polymyxin and other cationic antimicrobial peptides. To determine the effect of the mutation in BMM305 on susceptibility to polymyxin an overnight resistance assay was performed. Overnight cultures were grown in the usual way and supplemented with varying concentrations of polymyxin (Fig. 5.9). TT01 was able to grow overnight up to the highest concentration of polymyxin tested. However upon addition of 0.2μg/ml overnight growth was reduced by approximately 40%, and upon addition of higher amounts of polymyxin remained reduced by approximately 50% compared to growth in the absence of polymyxin. The overnight growth of BMM305 is also reduced upon addition of 0.2μg polymyxin. However, in contrast to TT01, BMM305 fails to grow at concentrations of polymyxin ≥0.4μg/ml, indicating that BMM305 is significantly more sensitive to polymyxin than TT01.

5.2.11 Resistance to Polymyxin is Adaptive

In *Salmonella* resistance to polymyxin is mediated through the two-component pathway PmrAB in response to environmental stimuli such as ferric iron (Wösten *et al.*, 2000; Gunn *et al.*, 2000). Furthermore, resistance to antimicrobial peptides can be induced by exposure to antimicrobial peptides (Bader *et al.*, 2003; McPhee *et al.*, 2003). In order to examine the response of *Photorhabdus* to exposure to antimicrobial peptides a growth curve was performed using both TT01 and BMM305 exposed to varying amounts of polymyxin. Both TT01 and

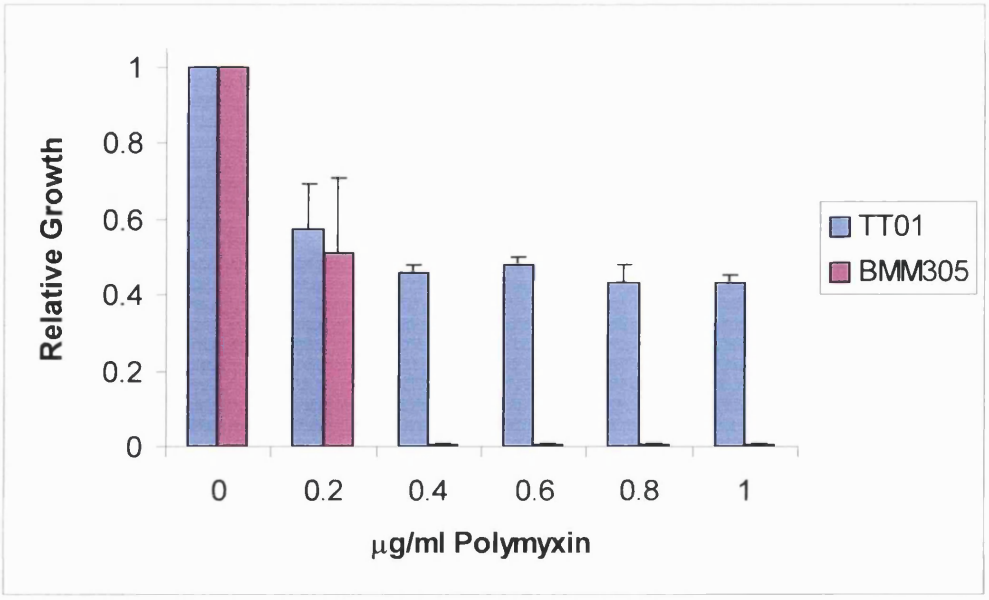
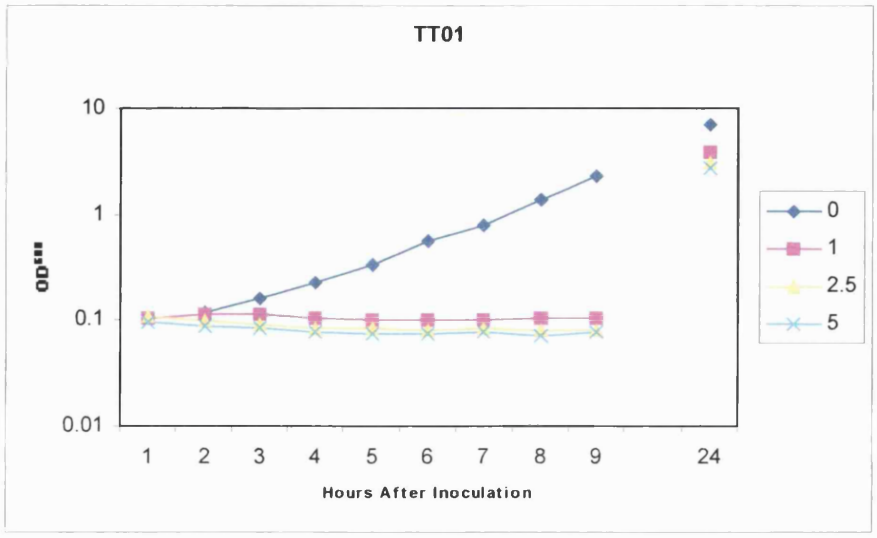


Figure 5.9. Relative growth (OD_{600}) of *Photorhabdus luminescens* TT01 (blue) and BMM305 (dark red), in different concentrations of polymyxin. Bars represent an average of three repeats and Y error bars represent standard deviation.

BMM305 were grown overnight and inoculated into conical flasks to approximately the same OD_{600}. Polymyxin was added to varying concentrations and the cultures incubated at 28°C and 200rpm and OD_{600} measurements were taken every hour (Fig. 5.10). In the absence of polymyxin both TT01 and BMM305 grew to an OD_{600} of 2.0 and 1.5 respectively, over a 9h period. In the presence of 1, 2.5 and 5µg polymyxin, TT01 stayed at the inoculating OD_{600} of approximately 0.1 for 9h. However, as expected (Fig 5.9), when left for 24h all the TT01 cultures, exposed to all the levels of polymyxin tested, grew overnight to an OD_{600} of 50% (OD_{600} 3.0) that of TT01 grown in the absence of polymyxin (OD_{600} 7.0). In the absence of polymyxin BMM305 reached an overnight OD_{600} of 4.6. However the cultures of BMM305 exposed to polymyxin start to decline in OD_{600} from the first hour, and continued to decline over 9h until all three cultures are at less than half the original inoculating OD_{600} of 0.1. When left for 24h these cultures dropped in OD_{600} even further to approximately 10-20%

A)



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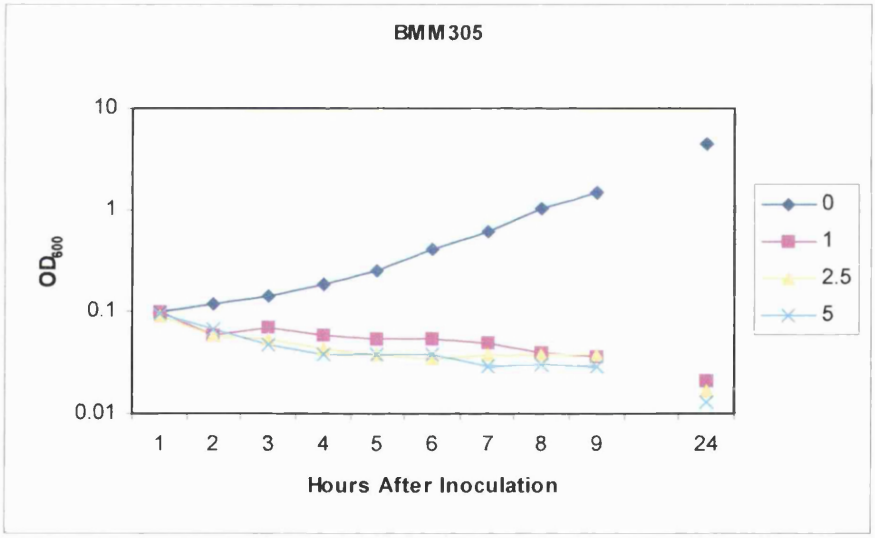

Figure 5.10. The growth of A) *Photorhabdus luminescens* TT01 and B) BMM305 in the presence of different concentrations of polymyxin B. Strains were inoculated from an overnight culture into conical flasks containing LB with the indicated concentration of polymyxin and grown at 28°C at 200rpm. Legend denotes μg polymyxin/ml

original inoculating OD_{600}. In conclusion TT01 appears to be resistant to killing by polymyxin and after a period of adaptation continues to grow. On the other hand, BMM305 cells appear to be immediately susceptible to killing by polymyxin and do not adapt or continue to grow.

5.2.12 Complementation of the Polymyxin Susceptible Phenotype

The mutant BMM305 was complemented with the various *pbgPE* genes and a qualitative assay for the restoration of resistance to polymyxin was performed. The strains TT01, BMM305/pTRC99a, pBMM500, pBMM501, pBMM502, pBMM503 and pBMM504 were grown overnight. The cultures were all resuspended in LB to an OD_{600} of 1.0 (equivalent to 10^8 CFUs/ml). A dilution series from 10^{-1} to 10^{-5} (10^7 to 10^3 CFUs/ml) was set up for each strain and 3µl of each of these cultures (equivalent to 30000 to 3 CFUs) were then pipetted, in duplicate, onto agar plates containing different levels of polymyxin. The large number of variables in this assay meant a plate-based assay would give easy visual comparisons.

The results show that TT01 was able to grow on the plates containing 0.1µg/ml polymyxin at all dilutions (Fig. 5.11). In the presence of 0.5µg/ml polymyxin only the 10^{-1} and 10^{-2} dilutions showed growth indicating the cells were not surviving well at this concentration. Interestingly, in liquid media TT01 is able to grow overnight in concentrations up to 1.0µg/ml polymyxin. This suggests the resistance of *Photorhabdus* to polymyxin is dependent on the growth conditions, i.e. planktonic or surface associated. In this assay BMM305 exhibited poor plating efficiency possibly due to the dilutant used for the dilution series, which was sterile distilled water. However even though BMM305 had a poor plating efficiency, the defect can be complemented by the presence of *pbgE1E2E3*, indicating it is either directly or indirectly due to the insertion.

On solid media BMM305 was able to grow in the presence of 0.1µg/ml polymyxin but growth only occurred at the 10^{-1} and 10^{-2} dilutions indicating very low levels of survival at this concentration (Fig. 5.10). However, at

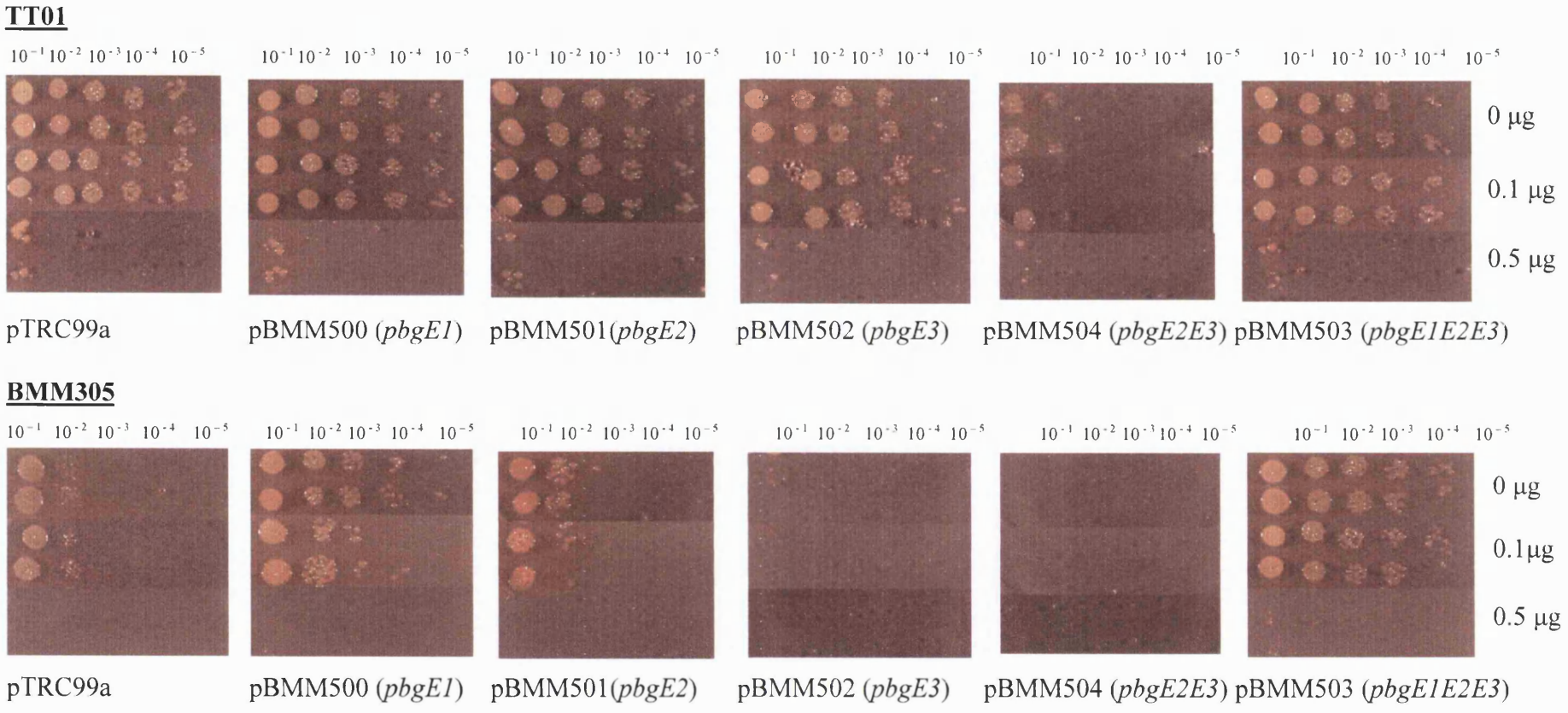

Fig 5.11. Plate assay showing the growth of *P. luminescens* TT01 and BMM305, containing *pbgPE* genes, when exposed to varying concentrations of polymyxin. Each strain was serially diluted and 3µl of each dilution were plate in duplicate.

0.5μg/ml there was no growth at the 10^{-1} dilution indicating no survival at this concentration. TT01/pBMM500, pBMM501, pBMM502 and pBMM503 were able to grow to the same degree as the parent strain. Interestingly, TT01/pBMM504 only showed growth at the 10^{-1} dilution suggesting that these genes have a detrimental effect on the survival of the cells. Only BMM305/pBMM501 grew to the same level as the parent strain; that is colony growth at 10^{-1} and 10^{-2}. BMM305/pBMM502 and pBMM504 failed to grow at any concentration of polymyxin and at any dilution. Interestingly BMM305/pBMM500 showed slightly better growth than the parent strain, with some colonies growing at the 10^{-4} dilution, and BMM305/pBMM503 showed growth similar to TT01 with a moderate number of colonies growing at the 10^{-5} dilution. Complementation of BMM305 with these genes failed to restore polymyxin resistance to TT01 levels, that is colony growth at 0.5μg/ml; however complementation with pBMM500 (*pbgE1*) did moderately restore the growth defect of the parent strain and complementation with pBMM503 (*pbgE1E2E3*) almost fully restored this defect. The complementation of polymyxin resistance may not be apparent due to the concentrations tested. The concentrations tested were based on resistance from liquid assays and should have been reassessed for solid based media. However this assay does demonstrate the only genes to complement a defect of BMM305 to be *pbgE1E2E3* and *pbgE1*.

In order to quantify the effect of complementation, an overnight relative growth polymyxin resistance assay was performed (Fig. 5.12). It has been previously shown that TT01 was able to grow at 0.4 and 1.0μg/ml polymyxin, whilst BMM305 failed to grow at 1.0μg/ml and at 0.4μg/ml (Fig 5.9). However, BMM305/pBMM500 was able to grow at 0.4μg/ml, reaching an overnight OD_{600} of approximately 60% the growth that occurred in the absence of polymyxin. Furthermore BMM305/pBMM503 was able to completely restore overnight growth to parental levels at 0.4μg/ml polymyxin. However both complemented strains failed to restore growth at 1.0μg/ml polymyxin. In conclusion, both *pbgE1* and *pbgE1E2E3* restored a growth defect of BMM305 on

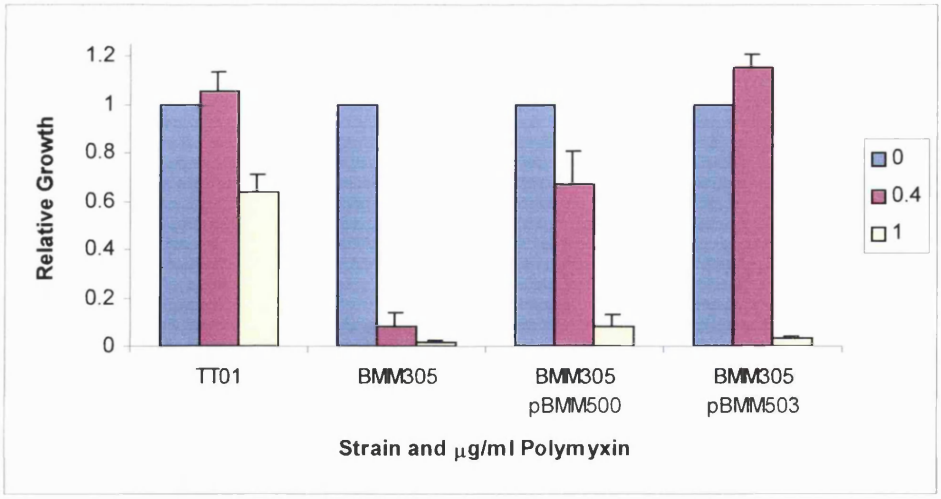

Figure 5.12. The relative growth (OD_{600}) of *Photorhabdus luminescens* TT01, BMM305, BMM305/pBMM500 (*pbgE1*) and BMM305/pBMM503 (*pbgE1E2E3*), in different concentrations of polmyxin, after overnight incubation at 28°C. Legend denotes μg/ml polymyxin. Bars represent an average of three repeats and Y error bars represent standard deviation.

solid media and partially restored polymyxin resistance in liquid media. This partial complementation of polymyxin resistance may be due to expression levels of the plasmid encoded genes. It may be that the genes encoded on the plasmid are not being expressed to appropriate parental levels and are therefore not conferring parental levels of polymyxin resistance.

5.2.13 BMM305 is Sensitive to Inorganic Acid

Photorhabdus is likely to encounter a wide range of external stresses as it changes host organisms. One such stress may be the pH inside the insect haemolymph, which is normally between pH 7 and pH 6 depending on the insect host and usually around pH 6.5 (S. Reynolds, personal communication). Interestingly an *E. coli* mutant lacking an O antigen was recently shown to be sensitive to acid stress (Barua *et al.*, 2002). Therefore, in order to determine the effect of pH on BMM305 an overnight relative pH growth assay was performed.

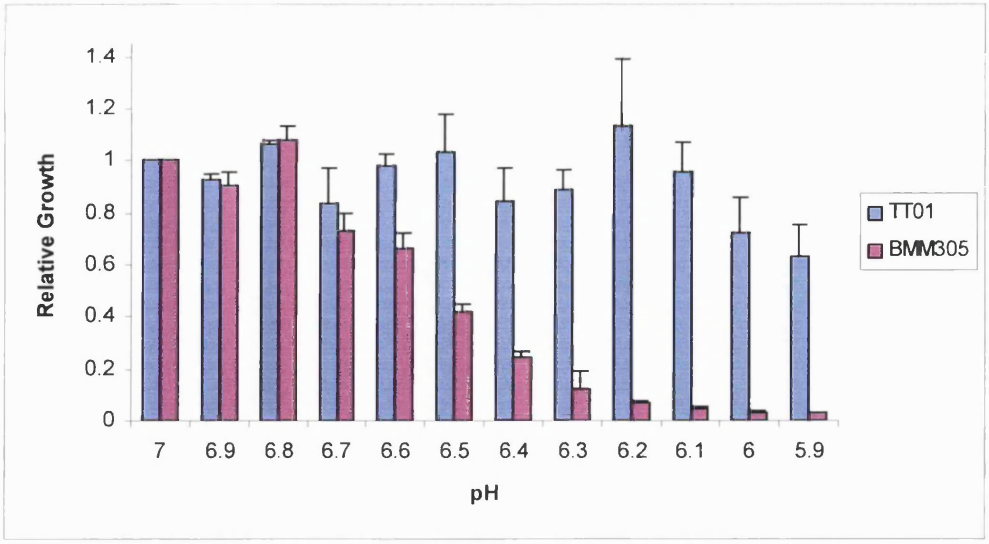

Figure 5.13. The relative growth (OD_{600}) of *Photorhabdus luminescens* TT01 (blue) and BMM305 (dark red), in differing concentrations of pH, after overnight incubation at 28°C. Bars represent an average of three repeats and Y error bars represent standard deviation.

Overnight cultures were prepared in the standard way; however the LB used was buffered with 50mM MES and the pH adjusted using HCl and NaOH to form a pH range of pH 7 to 6, in decreasing 0.1 increments (Fig. 5.13). These results clearly show that TT01 (compared to TT01 growth at pH 7) is able to grow over the whole pH range tested and the OD_{600} only started to drop at pH 6.0. In contrast, BMM305 (compared to BMM305 growth at pH 7) was able to reach an overnight OD_{600} similar to that of the growth at pH 7 until the pH was decreased to pH 6.6. However, from pH 6.5 and each increment thereafter, the overnight OD_{600} achieved by BMM305 dropped, until at approximately pH 6 when almost no overnight growth occurs. These results indicate that BMM305 is significantly more sensitive than TT01 to small changes in inorganic pH. *In vivo* acid stress is not just caused by H^+ but by organic acid as well. Organic acid not only lowers the pH of the medium but can also accumulate as intracellular anions inside the cell and cause internal damage (Barua *et al.*, 2002). To test the effect of organic

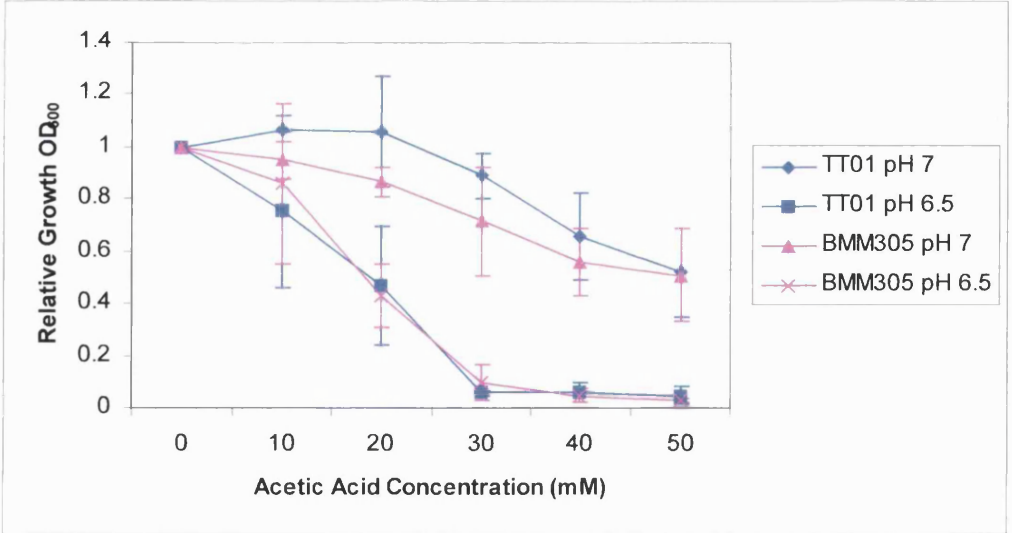

Figure 5.14. The relative growth of *Photorhabdus luminescens* TT01 and BMM305 in media buffered to pH 7 and pH 6.5, when exposed to various concentrations of acetic acid. Cultures were grown overnight with shaking at 28°C. Y error bars represent standard deviation over three repeats.

pH on BMM305 a similar overnight assay was performed with LB buffered with MES to pH 6.5 and pH 7 and to this media various concentrations of acetic acid were added (Fig. 5.14). After overnight growth in media buffered to pH 7 both TT01 and BMM305 continue to grow to almost normal levels until exposed to 30mM acetic acid at which point the overnight growth started to decline slowly. However even in the presence of 50mM acetic acid both strains still reached a minimum overnight relative OD_{600} of 50% the level of growth without acetic acid. The mutant BMM305 does appear to be slightly more susceptible to organic acid, at pH 7, than TT01 as it achieved a slightly lower level of relative growth at each concentration. However the trend in reaction to organic acid appeared to be similar and this difference in growth does not appear to be significant. After overnight growth in media buffered to pH 6.5 the OD_{600} attained by both TT01 and BMM305 started to drop slightly in the presence of 10mM acetic acid (to approximately 80-90% growth without acetic acid). This drop started to become significant in the presence of 20mM acetic acid as the

strains only achieved 30-50% normal growth. In the presence of 30mM acetic acid BMM305 achieved between 0 and 10% normal growth and TT01 achieved between 10 and 20% normal growth. Again, BMM305 appeared to be slightly more susceptible to organic acid than TT01 at pH 6.5; however the difference in overnight growth at any concentration of organic acid did not appear to be significant. The overall drop in growth upon increasing concentrations of organic acid at pH 6.5, for both strains, was significantly different from the growth that occurs at pH 7. These results suggest that the combined effect of increasing pH and organic acid has more of a detrimental affect on the cells than organic acid alone and, furthermore, that BMM305 is not significantly more sensitive to acetic acid than TT01.

5.2.14 Complementation of the Acid Susceptibility Phenotype

Cultures of BMM305/pBMM500 and pBMM503 were grown and an overnight liquid assay for the restoration of resistance to inorganic pH was performed (Fig. 5.15). At pH 6.5 BMM305 achieves an overnight OD_{600} of 30% the growth at pH 7 and at pH 6 BMM305 only achieves 10% of the growth observed at pH 7 (Fig 5.15). The presence of pBMM500 is able to complement growth at pH 6.5 and 6 to approximately 70% normal growth and the presence of pBMM503 is able to complement growth at pH 6.5 and 6 to approximately 80%. In conclusion the *in trans* expression of *pbgE1* and *pbgE1E2E3* complements the acid sensitive phenotype of BMM305.

5.2.15 PhoP Does Not Increase Resistance to Antimicrobial Peptides

In *Salmonella* the *pmrHFIJLKM* operon is regulated by the two-component pathway PmrAB (Gunn and Miller, 1996). Alternatively, PmrD can also regulate the pathway by posttranslationally activating the response regulator PmrA. The expression of *pmrD* is regulated in turn by the two-component pathway PhoPQ (Kato *et al.*, 2003). Therefore the activation of PhoPQ up regulates *pmrD,* which in turn activates PmrA, which increases transcription of the *pmrHFIJKLM* operon. However *in silico* analysis of the TT01 genome revealed no homology to *pmrA, pmrB, pmrD* or *pmrG* indicating an alternative method of regulation.

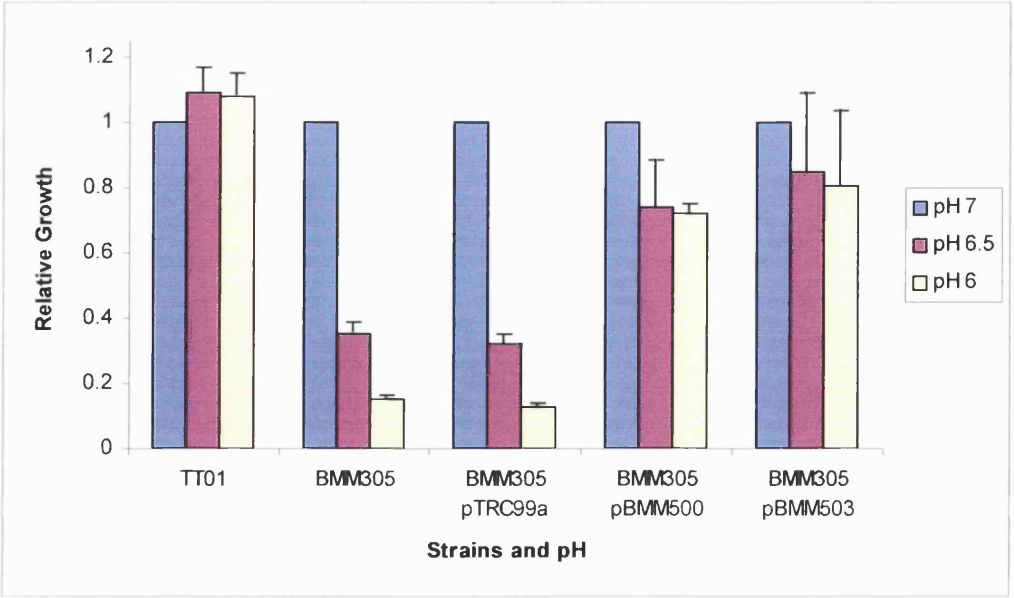

Figure 5.15. The relative growth (OD_{600}) of *Photorhabdus luminescens* TT01, BMM305, BMM305/pTRC99a, BMM305/pBMM500 (*pbgE1*) and BMM305/pBMM503 (*pbgE1E2E3*), in different concentrations of pH, after overnight incubation at 28°C. Bars represent an average of three repeats and Y error bars represent standard deviation.

However, TT01 does contain PhoP and Derzelle *et al.*, (2004a) have shown that *pbgE1* expression is regulated in response to Mg^{2+} in a PhoP dependent manner. Therefore polymyxin resistance may be mediated through the action of PhoP despite the absence of PmrAB. Furthermore it has previously been shown that the overproduction of PhoP can activate the target genes in a PhoQ independent manner (Lejona *et al.*, 2004). In order to determine whether PhoP was involved in polymyxin resistance the following experiment was performed. The response regulator PhoP was identified and amplified from the TT01 genome and cloned into the pTRC99a plasmid creating the plasmid pBMM505. A qualitative plate based assay for sensitivity of TT01/pBMM505 to polymyxin was performed. Cultures were grown and diluted as previously described and pipetted onto agar plates containing varying levels of polymyxin as previously described (Fig.

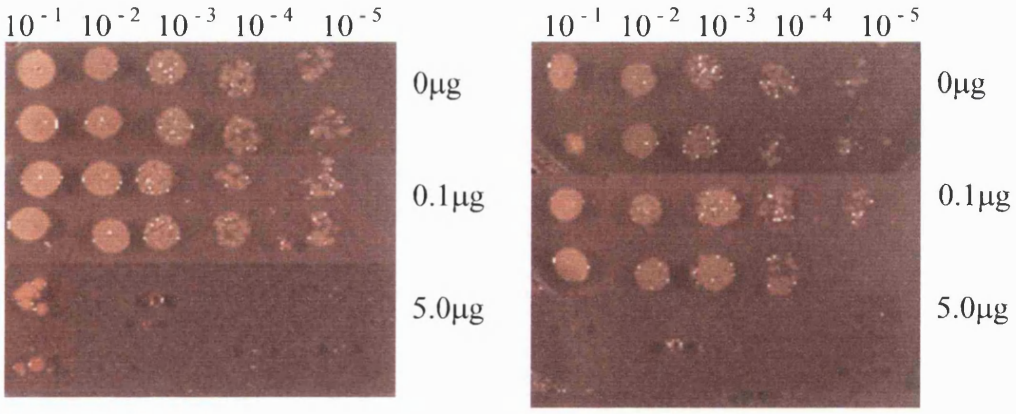

Figure 5.16. Plate assay showing the growth of *P. luminescens* TT01 pTRC99a and *P. luminescens* TT01 BMM505 when exposed to varying levels of polymyxin. Each strain was serially diluted and 3µl of each dilution were plated in duplicate at each concentration of polymyxin.

5.16). The strain TT01/pTRC99a shows growth at all dilutions when exposed to 0µg or 0.1µg and slight growth at 10^{-1} when exposed to 5.0µg polymyxin, as before (See Fig 5.11). However, TT01/pBMM505 shows slightly reduced growth at lower dilutions and no growth at 5.0µg polymyxin suggesting that over production of PhoP does not affect resistance to polymyxin. It may be that *phoP* is not expressed to the correct level to act in this plate assay. Lejona *et al.*, (2004) have shown that PhoP can also regulate its target genes in a PhoQ-independent manner when over expressed on a high copy number plasmid. It may be that pTRC99a is not a high enough copy plasmid to cause PhoQ-independent activation of PhoP and its target genes.

5.2.16 BMM305 is Sensitive to SDS

Bacteria that contain mutations in LPS genes tend to be more susceptible to membrane lysing agents such as lysozyme and various detergents (Raetz and

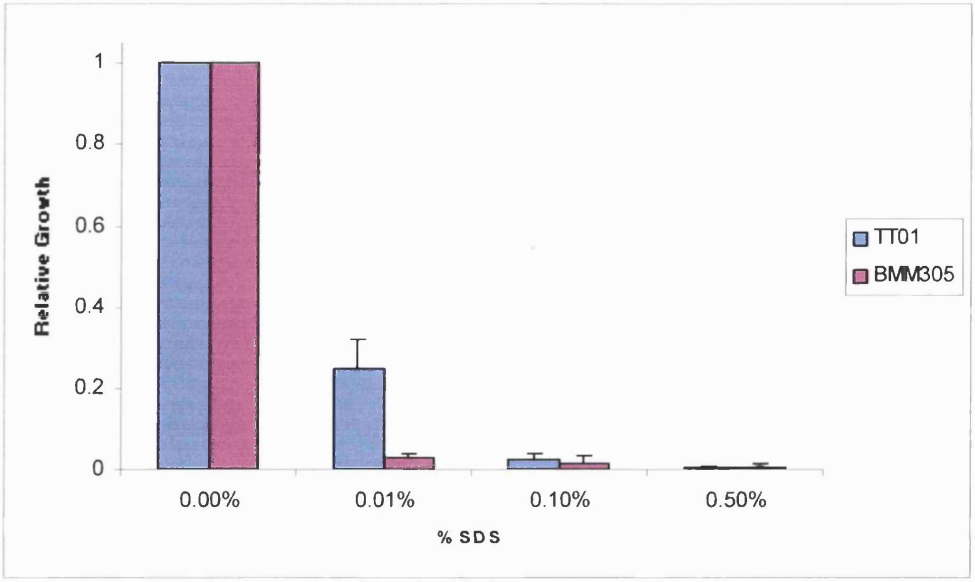

Figure 5.17. The relative growth (OD_{600}) of *Photorhabus luminescens* TT01 and BMM305 in different concentrations of SDS, after overnight incubation at 28°C. Bars represent average of three repeats and Y error bars represent standard deviation.

Whitfield, 2002). An overnight SDS susceptibility assay was performed on BMM305 to determine if the cells were more susceptible to such agents (Fig. 5.17). Both TT01 and BMM305 failed to grow overnight when exposed to 0.1% (w/v) SDS; however at a concentration of 0.01% (w/v) SDS, TT01 grew overnight to approximately 25% of the growth that occurred in the absence of SDS, whereas BMM305 grew to approximately only 5%. These results suggest that BMM305 may be more susceptible to SDS than TT01. The susceptibility of *Photorhabdus* to novobiocin was also assayed, however no measurable affect was observed (data not shown).

5.3 Conclusion

5.3.1 BMM305

Photorhabdus has a complex life cycle involving different hosts and changes in host interactions; becoming a symbiont then a pathogen and then a symbiont again (Forst and Clarke, 2002). These interactions must be accompanied by the

proper expression of genes needed for each respective interaction or *Photorhabdus* can become either avirulent or non-symbiotic (Derzelle *et al.*, 2004a; this study). Close bacterial associations with hosts can require physical contact and these are typically mediated through the appendages on the bacterial cell surface e.g. LPS. LPS is a good candidate for the study of the mediation of host interactions as it is highly variable in structure and can be easily modified.

The mutant BMM305 was isolated in a screen for motility defects and the insert was identified as having interrupted the gene *pbgE1* in the putative *pbgPE* operon. This operon has high homology with the *pmrHFIJKLM* operon in *Salmonella enterica*, which encodes enzymes involved in the synthesis and addition of aminoarabinoase ($_{L}$-Ara4N) to lipid A (Table 5.1). This suggested a similar role for the *pbgPE* operon in TT01, although several salient differences in the regulation of the two operons become apparent through *in silico* analysis. Most importantly the two-component pathway PmrAB is absent in the *Photorhabdus* genome; furthermore *pmrD* and *pmrG* do also not appear to be conserved. In *Salmonella* PmrD functions to activate PmrA and *pmrG* is regulated by PhoPQ via the PmrAB pathway. The absence of these genes that interact with or rely on PmrAB for expression further support the evidence for the absence of a PmrAB system from *Photorhabdus*.

The mutant BMM305 is approximately 20% less motile than TT01 and lacks O antigen, which is a component of LPS. Bacteria with defects in normal LPS structure or membrane protein components are well characterised as being susceptible to a wide range of stresses including membrane permeablising agents like surfactants and lysozyme, pH and antimicrobial agents (Ferguson *et al.*, 2002; Campbell *et al.*, 2002; Gunn *et al.*, 2000; Vaara, 1992). Defects in LPS have also been shown to affect flagella production i.e. deep rough mutants of *E. coli* are aflagellate (Parker *et al.*, 1992). Therefore disruptions in LPS have a precedent for affecting cell motility. Further to this, a mutation in *pmrK* in *Salmonella* was shown to prevent swarming; possibly due to the fact the LPS from the cells, which has been shown to be important in swarming, could no longer help to overcome surface tension and improve wettability of the medium (Kim *et al.*, 2003; Toguchi *et al.*, 2000). *Proteus* mutants that could not add

aminoarabinose to their LPS and were sensitive to polymyxin also could not swarm, further indicating the importance of correct LPS for motility (McCoy *et al.*, 2001). Although BMM305 is able to swarm it is affected in normal swimming motility; this indicates that correct LPS is not essential for *Photorhabdus* swarming although it is required for swimming. The role LPS may play in swimming in *Photorhabdus* may be similar to the role is has in swarming in other bacteria, i.e. affecting the medium through which the bacterium is passing. Interestingly, a *phoP* mutant in *Photorhabdus* is more motile than its parental strain indicating that PhoP may also act to repress motility (Derzelle *et al.*, 2004a). This further suggests LPS modifications and motility may be linked.

5.3.2 Synthesis of $_{L}$-Ara4N in *Salmonella*

The sugar UDP-glucose is converted to UDP-glucuronic acid by UDP-glucose dehydrogenase, which is encoded by *ugd* (Table 5.1). This is the substrate for the products of the *pmrHFIJKLM* operon. The product of the third gene in the operon *pmrI* catalyses an oxidative decarboxylation to UDP-4-keto-pyranose, and the product of the first gene *pmrH* catalyses a transamination, the transfer of an amino group (NH_2), to the 4' position to create UDP-$_{L}$-Ara4N. The gene *pmrF* is the second gene in the operon and in *Salmonella* encodes a sugar transferase that is likely to transfer the $_{L}$-Ara4N moiety to undecaprenyl phosphate, a lipid based carrier molecule, to form undecaprenyl phosphate-α-$_{L}$-Ara4N. This is then translocated to the outer surface of the inner membrane where the product of the fifth gene, *pmrK*, which is a predicted mannosyltransferase protein, acts to transfer the $_{L}$-Ara4N moiety to lipid A (Baker *et al.*, 1999; Raetz and Whitfield, 2002).

The *Salmonella* genes *pmrJ*, *pmrL* and *pmrM* have not yet been assigned biochemical functions in the synthesis of $_{L}$-Ara4N or its transfer to lipid A. However, non-polar deletion analysis demonstrated that *pmrHFIJKL* are all essential for addition of $_{L}$-Ara4N and subsequent resistance to polymyxin (Gunn *et al.*, 2000). In *Salmonella* however *pmrM* is not required and addition of $_{L}$-Ara4N is visible by analysis of lipid A from this mutant strain (Gunn *et al.*, 2000). The homologues of *pmrJ*, *pmrL* and *pmrM* in *Photorhabdus* are *pbgP4*,

Production of lipid A-$_L$-Ara4N ⟶	**Substrate**	**Gene in *Salmonella***	**Gene in *Photorhabdus***	**Predicted *Photorhabdus* protein homology**	**Reaction**	**Product**
	UDP-glucose	*ugd*	*ugd* (*plu2500*)	UDP-glucose dehydrogenase	dehydrogenase	UDP glucuronic acid
	UDP glucuronic acid	*pmrI*	*pbgP3*	Putative formyl transferase	oxido-reductase	UDP-4-keto-pyranose
	UDP-4-keto-pyranose	*pmrH*	*pbgP1*	Putative transaminase	transamination	UDP-$_L$-Ara4N
	UDP-$_L$-Ara4N	*pmrF*	*pbgP2*	Putative glycosyl transferase	transfer	undecaprenyl phosphate-α-$_L$-Ara4N
	undecaprenyl phosphate-α-$_L$-Ara4N	*pmrK*	*pbgP5*	Dolichyl-phosphate-mannose-protein (mannosyltransferase)	transfer	**lipid A-$_L$-Ara4N**
		pmrJ	*pbgP4*	Hypothetical protein	unknown	
		pmrL	*pbgE2*	Putative membrane protein	unknown	
		pmrM	*pbgE3*	Putative membrane protein	unknown	

Table 5.1. Genes of the *pbgPE* operon in *Photorhabdus*, their homologues in *Salmonella* and the predicted protein products in *Photorhabdus*. Table also includes the substrates and products of the putative reactions of the protein products, starting from UDP-glucose (bold) to lipid A -$_L$-Ara4N (bold).

pbgE2 and *pbgE3* respectively. The gene *pbgP4* encodes a hypothetical protein and *pbgE2* and *pbgE3* encode small putative membrane proteins with homology to each other.

In *Salmonella* the *pmrHFIJKLM* operon is under the transcriptional control of the two-component pathway PmrAB where PmrB is the sensor kinase and responds to high Fe^{3+} and activates PmrA, which is the response regulator and binds to DNA to promote transcription (Wösten *et al.*, 2000). Interestingly a constitutive *pmrA* mutant had higher amounts of $_{L}$-Ara4N and phosphoethanolamine modified lipid A. The addition of phosphoethanolamine to lipid A has also been suggested to be under the control of the PmrAB two-component pathway (Gunn *et al.*, 1998). In *Salmonella* an adjacent gene *pmrG* is also regulated by PmrAB yet has no function $_{L}$-Ara4N modifications or in specific resistance to polymyxin (Gunn *et al.*, 1998). It is interesting to speculate whether *pmrM* or *pmrG* may be involved in modification of LPS with phosphoethanolamine in *Salmonella.*

5.3.3 *Photorhabdus pbgPE* Operon

The control of the expression of the *Photorhabdus pbgPE* operon is mediated by Mg^{2+} in a PhoPQ dependent manner, as is the *pmrHFIJKLM* operon in *Salmonella* (Derzelle *et al.*, 2004a; Gunn and Miller, 1996). Furthermore, modifications to the core/lipid A region of *Photorhabdus* LPS can be visualised in the presence of low concentrations of Mg^{2+} (Derzelle *et al.*, 2004a). Therefore it is likely that the *pbgPE* operon in *Photorhabdus* encodes proteins involved in the synthesis and addition of $_{L}$-Ara4N to lipid A in a manner similar to *Salmonella.*

The *pmrHFIJKLM* mediated modification in *Salmonella* LPS is relatively small (the addition of one or two $_{L}$-Ara4N groups) and a *pmrK* mutant produces only subtle differences in LPS structure. However, in *Photorhabdus* the effect was significant and BMM305 was shown to lack the normal O antigen of LPS. This phenotypic difference highlights the genetic disparity between *Photorhabdus* and *Salmonella* and suggests a novel role for the putative *pbgPE* operon in *Photorhabdus*. It is unclear how the *pbgE1* mutation results in a lack of O

antigen or if this strain also lacks some core regions. However from complementation analysis it is apparent that the downstream genes *pbgE2E3* are vital for correct O antigen expression (Fig. 5.4). Therefore in *Photorhabdus* the genes *pbgE2E3* may not have a role in $_{L}$-Ara4N synthesis but may have other functions in LPS synthesis. Alternatively, these genes may have dual roles and are involved in $_{L}$-Ara4N synthesis in an, as yet, unknown manner as well as O antigen production. This has precedence in the *pmrI* gene product of the *Salmonella pmrHFIJKLM* operon, which has two active domains; one which catalyses oxidative decaboxylation in the synthesis pathway of $_{L}$-Ara4N, and one which can transfer a formyl group to UDP-$_{L}$-Ara4N at a slightly later stage in the pathway although the significance of this modification is not clear (Raetz and Whitfield, 2002).

The genes *pbgE2E3* are predicted to encode small putative membrane proteins with homology to each other. It is unknown how they act to effect the completion of correct O antigen structure but complementation of BMM305 with *pbgE1* alone does not restore the O antigen, and neither does complementation with *pbgE2E3* without *pbgE1* (data not shown). This indicates that the whole operon is required and the putative addition of $_{L}$-Ara4N to lipid A may be an essential step in LPS biosynthesis in *Photorhabdus* (Table 5.2).

O antigen is assembled on the cytoplasmic side of the inner membrane where three membrane proteins Wzx, Wzy and Wzz work to add repeating sugar units to a growing O antigen present on the periplasmic side (for a review see Raetz and Whitfield, 2002). Although PbgE2 and PbgE3 do not have homology to these proteins, *in silico* analysis does suggest that they are themselves membrane proteins. Therefore they may interact with Wzx, Wzy or Wzz to affect the transport of O antigen across the membranes or the ligation of O antigen to the core region.

The over-expression of *pbgE2E3* resulted in apparent poor survival of TT01 and BMM305 on solid media (Fig. 5.11). However, as the strains had been grown on solid and liquid LB based media with no apparent problem it is possible the over

Strain	*pbgPE* genes putatively transcribed							O antigen?
TT01 (parent)	*P1*	*P2*	*P3*	*P4*	*E1*	*E2*	*E3*	**Yes**
BMM305 (mutant)	*P1*	*P2*	*P3*	*P4*				**No**
BMM305 pBMM500	*P1*	*P2*	*P3*	*P4*	*E1*			**No**
BMM305 pBMM503	*P1*	*P2*	*P3*	*P4*	*E1*	*E2*	*E3*	**Yes**
BMM305 pBMM304	*P1*	*P2*	*P3*	*P4*		*E2*	*E3*	**No**
	Synthesis and addition of $_L$-Ara4N					**Completion of O antigen**		

Table 5.2. Genes of the *pbgPE* operon putatively transcribed in TT01, BMM305 and BMM305 complemented with combinations of genes. Right hand column indicates the presence or absence of O antigen in each strain and the bottom row indicates putative functions of above genes.

expression of *pbgE2E3* resulted in poor survival in the diluting agent, which was sterile distilled water. Both *pbgE2* and *pbgE3* encode putative membrane proteins, and it is possible the presence of these genes on a plasmid leads to the alteration and/or disruption of the membrane composition and susceptibility to osmotic pressure.

5.3.4 BMM305 and Host Stresses

The mutant BMM305 was more susceptible to decreases in inorganic pH and also to the antimicrobial peptide polymyxin B. The increase in susceptibility to antimicrobial peptides is well documented for mutants of the *pmrHFIJKLM* operon in *Salmonella* (Gunn *et al.*, 2000). In *Salmonella* the addition of $_L$-Ara4N is an adaptive response and probably occurs on the outer surface of the inner membrane during the process of LPS synthesis (Raetz and Whitfield, 2002). Therefore novel $_L$-Ara4N containing LPS molecules must be synthesised before resistance arises. The resistance of *Photorhabdus* to polymyxin also appears to

be adaptive (Fig. 5.10) although several differences arise in the regulation of resistance in *Salmonella* and *Photorhabdus* and have been highlighted (This study; Derzelle *et al.*, 2004a).

5.3.5 PhoP and the *pbgPE* Operon in *Photorhabdus*

The expression of *pbgP1*, the first gene of the *pbgPE* operon, is regulated by Mg^{2+} concentration; however this regulation is abolished in a *phoP* mutant therefore suggesting PhoP mediated expression of the *pbgPE* operon (Derzelle *et al.*, 2004a). In *Salmonella* PhoP recognises a region upstream of the transcription start site of its target genes termed the PhoP box. This box consists of a direct repeat of the heptanucleotide sequence (T)G(T)TT(AA) (Yamamoto *et al.*, 2002). However there is no obvious PhoP box in the region upstream of the putative start site of *pbgP1* suggesting PhoP activation may be indirect (Derzelle *et al.*, 2004a). Moreover, in *Salmonella* PhoPQ regulates the gene *pagP* in a Mg^{2+} limited environment (Bishop *et al.*, 2000). Although *Photorhabdus pagP* also appears to be regulated in response to Mg^{2+} limitation it does not appear to be dependent on PhoPQ (Derzelle *et al.*, 2004a). Finally, the *Photorhabdus phoP* mutant was not attenuated for growth in low concentrations of Mg^{2+} unlike the *Salmonella phoP* mutant, which further suggests divergent roles for PhoPQ in *Photorhabdus* (Sonicini *et al.*, 1996).

These differences in PhoP regulation and the absence of PmrAB suggest that the *pbgPE* operon, although homologous to the *pmrHFIJKLM* operon, may also be differentially regulated in *Photorhabdus* and therefore have a different significance in the life cycle of *Photorhabdus.* In support of this, PhoPQ is two-component pathway normally associated with growth in Mg^{2+} limiting conditions, such as intracellular growth within a macrophage, yet intracellular growth is not known to be a key factor in a *Photorhabdus* infection of an insect host. Furthermore TT01 is relatively resistant to polymyxin with a MIC of greater than 250μg/ml, whereas a $PmrA^{C}$ *Salmonella* strain had a MIC of just 4μg/ml (compared to *Salmonella* $PmrA^{+}$ MIC 1μg/ml) (Derzelle *et al.*, 2004a; Gunn *et al.*, 2000; Vaara, 1992). The differences in natural resistance could be due to differences in general LPS structure and charge; alternatively this may

further suggest differences in regulation of *pmrHFIJKLM/pbgPE* resulting in different MICs. One hypothesis for the difference in MICs is that the *pbgPE* operon of *Photorhabdus* is constitutive or positively regulated under a wider range of environmental cues and the LPS may therefore be more heavily substituted with $_{L}$-Ara4N and perhaps phosphoethanolamine.

5.3.6 BMM305 and Virulence

BMM305 was highly attenuated in virulence; BMM305 complemented with *pbgE1* was partially resistant to polymyxin (Fig. 5.12) and was able to grow at acidic pH (Fig. 5.15). However when injected into insect larvae BMM305/pBMM500 was still attenuated in virulence (Fig. 5.8). This suggests that resistance to CAMPs and pH is not essential in *Photorhabdus* virulence.

Furthermore BMM305 lacks an O antigen and this is only restored when complemented with *pbgE1E2E3*. This complemented strain is also resistant to CAMP and acidic pH and is able to kill insect larvae in numbers comparable to TT01 (Fig.5.8). In summary these data correlate the presence of the full *pbgPE* operon with O antigen and virulence, whereas the presence of only *pbgP1P2P3P4E1* (and consequently the modification of lipid A) is necessary for antimicrobial peptide and acid resistance (Table 5.3).

The presence of structurally correct *Photorhabdus* LPS can contribute towards virulence in a number of ways. 1) LPS provides resistance to CAMPs, complement and other host derived antimicrobial compounds (Guo *et al.*, 1998; Gunn *et al.*, 2000; Taylor, 1995; Waldor *et al.*, 1994). 2) LPS damages haemocytes (Dunphy and Hurlbert, 1995; Dunphy and Webster, 1988). 3) LPS inhibits the activation of prophenoloxidase and therefore prevents melanisation (Dunphy, 1995). 4) LPS affects cell hydrophobicity and charge and subsequently affects interactions with, and attachment to, specific haemocytes (Dunphy and Hurlbert, 1995).

The attenuated virulence phenotype of BMM305 is putatively due to the lack of O antigen and not to the susceptibility of BMM305 to antimicrobial peptides

Gene	*pbgP1*	*pbgP2*	*pbgP3*	*pbgP4*	*pbgE1*	*pbgE2*	*pbgE3*
Function	Synthesis and addition of $_{L}$-Ara4N					Completion of O antigen	
Phenotype	Resistance to antimicrobial peptides Resistance to mildly acidic conditions					Virulence	

Table 5.3. Summary of the genes of the *pbgPE* operon, their functions and phenotypes.

produced by the insect innate immune response or acidic pH. However the relative levels of CAMPs and exact pH in the insect haemolymph are unknown, and physiological conditions inside the haemolymph may act to increase the action of certain CAMPs or other antimicrobial actions. For example, pre-exposure to polymyxin sensitises *E. coli* to other antimicrobial compounds and increased acidic pH in the presence of organic acids increases *E. coli* susceptibility to complement (Vaara, 1992; Ocaña-Morgner and Dankert, 2001). Therefore, *in vivo* conditions may combine and exacerbate their actions on BMM305. Consequently, although the presence of *pbgE1 in trans* in BMM305 restores resistance to polymyxin, *in vitro* a more robust outer membrane including O antigen may be needed to resist other antimicrobial agents in the insect haemolymph. Although this data suggests resistance to CAMPs and acidic pH are not essential for virulence a clearer understanding of the composition of insect haemolymph and response of BMM305 carrying *pbgE1* to these components is needed.

Although purified *Photorhabdus* LPS is non-lethal to insects upon injection (Clarke and Dowds, 1995) there is evidence to suggest that *Photorhabdus* LPS may have a role in pathogenicity. The release of *Photorhabdus* LPS triggered by larval serum has been shown to correlate to haemocyte damage and survival in the haemolymph has been partially attributed to the ability of *Photorhabdus* to destroy haemocytes (Dunphy and Webster, 1988; Dunphy, 1995; Au *et al.*, 2004). Therefore LPS may play a direct role in survival in the haemolymph.

Furthermore four avirulent transpositional mutants of *Xenorhabdus* were found to exhibit rough LPS profiles although the sites of insertion were not identified (Dunphy and Hurlbert, 1995).

Dunphy (1995) suggested that the presence of LPS inhibits phenoloxidase. Interestingly, injection of high numbers of BMM305 caused insect death and the cadavers turned dark brown in colour (data not shown). This is in contrast to TT01 killed larvae which turn brick red (data not shown). This indicates the presence of melanin and the activation of prophenoloxdase, which correlates with the absence of correct LPS. BMM305 when co-injected into insect hosts in equal numbers with TT01 failed to grow to detectable numbers indicating the attenuated phenotype could not be rescued by the presence of *in trans* O antigen. Interestingly this suggests the attenuated phenotype is due to a failure of BMM305 to grow rather than a failure to elicit cellular damage and immune suppression/resistance.

Although complementation of BMM305 with *pbgE1E2E3* did restore cell dosage based virulence to approximate parental levels (100 cells gave ~100% mortality) it failed to restore the normal time it took to kill (96h as opposed to 48 for TT01). This could be due to some BMM305 cells losing the plasmid carrying *pbgE1E2E3*. As the insect haemolymph does not contain any antibiotic selection pressure these cells would then revert to the avirulent phenotype effectively reducing the number of virulent *Photorhabdus* cell present. This reduction in numbers of virulent cells would effectively cause a growth lag and an increase in LT_{50}. Alternatively the growth rate of BMM305 carrying the plasmid may be affected *in vivo*, even though it is unaffected *in vitro*, and as growth rate has been correlated to virulence this may explain the increased LT_{50} (Clarke and Dowds, 1995).

5.3.7 BMM305 and Symbiosis

The symbiosis of *Photorhabdus* with *Heterorhabditis* can be divided into two stages (Forst and Clarke, 2002). The first stage involves supporting the growth and development of the nematode partner and the second stage involves successful colonisation of the IJ intestine. The mutant BMM305 is clearly

affected in stage II of symbiosis. Little is known about the interactions of *Photorhabdus* with its symbiotic host *Heterorhabditis*; how *Heterorhabditis* retains the bacteria, the cellular interactions and signals involved and lastly what active or inactive role *Photorhabdus* has in this symbiosis. Recent developments in understanding the parallel system in *Xenorhabdus* have suggested the IJs retain a very small number of cells, which then grow to form the final population (Martens *et al.*, 2003). Initial experiments with *Photorhabdus* suggest a similar mechanism for colonising *Heterorhabditis* IJs (R. Watson, personal communication). Therefore BMM305 may be attenuated in either the ability to colonise the intestine or the ability to grow and form the final population.

5.3.7.1 Stage I - LPS in Colonisation

Correct O antigen structure is essential for the symbiosis of *Rhizobium* with its plant host and mutants with defects in correct LPS structure have been shown to fail at various stages of symbiosis (Noel *et al.*, 1986; Campbell *et al.*, 2002). Furthermore, LPS from *Rhizobia* have been shown to vary in structure and characteristics depending on the source of isolation; free-living bacteria or bacteroids (Sindhu *et al.*, 1990; Kannenberg and Carlson, 2001). Therefore *Rhizobial* LPS has a significant and active role in the symbiosis with plants and may act as a focal point for the plant to recognise the bacteria as symbionts (Perotto *et al.*, 1994). Unlike most bacterial surface structures (flagella, pili) the structure of LPS has the capacity to be highly variable in size and composition leading to an extremely adaptable structure that can influence bacterial properties such as surface charge, hydrophobicity and subsequently cell adhesion and cell-cell interactions (Dunphy, 1995; Toguchi *et al.*, 2000). Therefore LPS represents an appealing cell membrane component for potentially mediating both pathogenic and symbiotic host interactions.

The *pbgE1* gene in *Photorhabdus* has been shown to be important in the production of O antigen, which is required for the correct colonisation of the nematode host. The O antigen may represent a distinguishing feature necessary for recognition of the bacterial partner. In *Rhizobium leguminosarum* it has been suggested there is a balance between eliciting a symbiotic or pathogenic interaction between plant and bacterium, and the defects in LPS in mutant strains

accentuates the non-recognition of symbiont and subsequent host defence response (Perotto *et al.,* 1994). In the same way *Heterorhabditis* IJs may not recognise BMM305 as a symbiont and therefore not retain it.

LPS isolated from bacteroid *Rhizobium* cells has also been shown to be more hydrophobic than LPS from free-living cells (Kannenberg and Carlson, 2001). The authors suggest this may mediate attachment by allowing non-specific close interactions of the bacteroid with the plant membrane. In the same way, *Photorhabdus* mutants with altered cell surface hydrophobicity were shown to bind to insect haemocytes in a manner different from the parental strain (Dunphy, 1995). Moreover, TEM has shown that *Photorhabdus* cells do have intimate contact with the nematode gut epithelium (ffrench-Constant *et al.*, 2003). Therefore LPS may mediate these close associations via generation of non-specific hydrophobic bacterial-host cell interactions.

5.3.7.2 Stage II - LPS in Survival and Growth Inside the Nematode

The environmental conditions experienced by bacteroids are very different from those experienced by free-living *Rhizobia.* As the fixation of nitrogen is sensitive to the presence of oxygen the bacteroids are under low-oxygen and possibly slightly acidic conditions. In support of this LPS isolated from *Rhizobium* grown *in vitro* under low oxygen or slightly acidic conditions had characteristics associated with LPS isolated from bacteroids. Therefore *Rhizobia* have the capacity to alter their LPS depending on environmental conditions. A *Sinorhizobium* mutant with an altered core region showed sensitivity to CAMPs and failed to support symbiosis; the author suggested that sensitivity to the hosts innate immune response contributed to the failure to sustain chronic infection (Campbell *et al.*, 2002). Therefore the ability to sense and respond to changes in the environment is critical to the successful symbiosis of many *Rhizobia* and these changes in the environment are mirrored in changes in LPS composition and character.

The environmental conditions inside the intestine of the IJ stage of both *Heterorhabditis* and *Xenorhabdus* nematodes are unknown. The transcriptional regulator σ^S mediates the response to a wide variety of stresses including

osmolarity, acid shock, heat shock, starvation and the onset of stationary phase (Hengge-Aronis, 2000). Furthermore σ^S has been shown to be important in the host interactions of other bacteria largely through controlling responses to host mediated stresses (Yildiz and Schoolnik, 1998). The sigma factor σ^S is encoded by the gene *rpoS* and a *Xenorhabdus rpoS* mutant was shown to be defective in colonisation of the nematode host *Steinernema* (Vivas and Goodrich-Blair, 2002). Together these results suggest the possible presence of nematode mediated stress that the *Xenorhabdus* mutant cannot overcome. The presence of LPS in bacteria has been shown to provide resistance to stresses such as acid (Barua *et al.*, 2002), CAMPs (Gunn *et al.*, 2000) and complement (Taylor, 1995). We have shown that BMM305 lacks an O antigen and is susceptible to stresses such as the membrane damaging agent SDS, polymyxin and acidic pH. The lack of successful symbiosis with the nematode may therefore be due to the failure of BMM305 to survive and grow within the intestine due to the presence of host mediated stresses.

5.3.8 LPS as a Signal Molecule

It has been suggested that *Rhizobial* LPS may act as a signal molecule that induces developmental changes, necessary for symbiosis, in both the bacterium and the plant (Campbell *et al.*, 2002; Perotto *et al.*, 1994). Indeed, LPS alone has been demonstrated to induce structural changes in the plant including the stimulation of infection thread formation (Dazzo *et al.*, 1991). *Photorhabdus* LPS has been shown to be released during growth and to interact with insect host cells inducing damage (Dunphy, 1995). It is possible that LPS is released from *Photorhabdus* inside the nematode and acts as a signal that interacts with host cells to elicit a symbiotic response. In this hypothesis the LPS from BMM305 would represent an altered signal, perhaps unrecognisable by the nematode thus leading to failed symbiosis. However, if this hypothesis is correct the presence of parental *Photorhabdus* with the correct LPS signal should rescue BMM305. However, nematodes grown on TT01 and BMM305 in equal proportion were shown to retain only TT01 or to retain BMM305 in numbers so small as to be undetectable. This suggests that if LPS does act as a symbiotic signal the signal is only one part of the factors necessary for complete colonisation.

5.3.9 The Gene *pbgE1* is Required for the Colonisation of Both *Photorhabdus* Host Organisms

The *pbgPE* operon has been shown in this study to be important for the production of the O antigen component of LPS. The O antigen of *Photorhabdus* is required for the successful colonisation of both the insect host and the nematode host. The involvement of O antigen (and therefore *pbgE1*) in this overlap in host interactions represents the first gene of *Photorhabdus* to be identified as being involved in both symbiosis and pathogenicity. It has been suggested that bacterial-host associations are a continuum of interactions from virulence to mutualism (Hentshel *et al.*, 2000; Paracer and Ahmadjian, 2000). Moreover many bacteria use similar mechanisms for infection resulting in either virulent or symbiotic interactions. A common mechanism includes the use of two-component pathways, which are essential for recognising and responding to environmental cues and can respond by activating or repressing symbiosis or virulence genes. For example, PhoPQ is essential for virulence in *Photorhabdus* (Derzelle *et al.*, 2004a) and the two-component sensor RscS of *Vibrio* is required for symbiosis (Visick and Skoufos, 2001). Furthermore flagella, motility and LPS can also facilitate both symbiotic and pathogenic interactions (Otterman and Miller, 1997; Fraysse *et al.*, 2003; Morona *et al.*, 2003). Another mechanism shared by symbionts and pathogens is horizontal gene transfer for the acquisition of genes resulting in either symbiosis or pathogenicity islands respectively (Sullivan *et al.*, 1995; Groisman and Ochman, 1997).

This study demonstrates that *Photorhabdus* O antigen serves as an essential requirement for both symbiosis and pathogenicity in two different hosts. Therefore it is in the context of the host organism that virulence or symbiosis is elicited. Moreover *Photorhabdus* can occupy both ends of the host interaction continuum and understanding how it can shift from pathogen to symbiont and the molecular mechanisms involved provides a useful model for bacteria with less tractable host organisms.

Interesting inverse parallels can be drawn from another bacteria/nematode interaction: the pathogenic *Salmonella/Caenorhabditis elegans* model. In this model *Salmonella* colonises the intestine of *C. elegans* and eventually kills the

nematode (Aballay *et al.*, 2000). Interestingly, the presence of a complete O antigen is necessary for full virulence of *Salmonella* towards *C. elegans* as is the two-component pathway PhoPQ (Aballay *et al.*, 2003: Aballay *et al.*, 2000). In *Photorhabdus* a complete O antigen is required for symbiosis and PhoPQ controls the expression of the *pbgPE* operon. Furthermore *Salmonella rpoS* mutants were also attenuated in virulence in the *C. elegans* model host (Labrousse *et al.*, 2000) and *rpoS* has been shown to be important in *Xenorhabdus* colonisation of *Steinernema* (Vivas and Goodrich-Blair, 2002). Therefore the *Photorhabdus/Heterorhabditis* and *Salmonella/C. elegans* associations represent similar models using both *Enterobacteriaceae* and *Rhabditid* nematodes, which interact using parallel mechanisms with remarkably different outcomes. Further study of these systems should yield interesting findings into the nature of symbiosis and pathogenicity from the prospective of both nematode and bacterium.

CHAPTER 6

6.1 General Discussion

The aim of this study was to try and elucidate the mechanisms used by *Photorhabdus* in the symbiotic interaction with its nematode host and pathogenic interaction with its insect host. The approach used was one of random transposon mutagenesis and screens based on the hypothesis that motility, specifically flagella based motility, has a role in these interactions. Flagella mediated motility was found not to be essential for virulence towards *G. mellonella*, although it was important for the colonisation of the nematode host. However, in order to fully understand the role of flagella mediated motility in symbiosis, flagella and motility need to be decoupled in a mutant strain that has complete flagella, which do not rotate and is therefore non-motile. This would discover the precise role of flagella in symbiosis; whether they are needed for attachment or for motility. Furthermore the mutation in *flgG* should be complemented with *flgG* and the downstream genes to ensure that the phenotype seen in BMM316 is due to this mutation and not pleiotropic effects on other genes.

As nematodes grown on BMM316 have been shown to retain significantly fewer cells and even less when grown on an equal mix of BMM316 and TT01 it would be interesting to study this effect over several generations of the nematode life cycle. One hypothesis might be that if grown on BMM316 and repeatedly passaged through insect hosts a low basal level of BMM316 would persist such as seen after one passage through *G. mellonella*. However, if grown on an equal mix of TT01 and BMM316 and repeatedly passaged through insect hosts another hypothesis would be that BMM316 would eventually be out competed and numbers would fall to undetectable levels. This experiment would allow the exploration of the idea that motility offers a significant competitive advantage and *Photorhabdus* is motile through selective pressure.

Silva *et al.*, (2002) studied the bacterial infection of *Photorhabdus* in a different insect host, *Manduca sexta*, and these results suggested a role for bacterial motility *in vivo*. Although motility is not important in the pathogenicity of *Galleria mellonella* it would be interesting to test the virulence of BMM316 in other insect hosts to demonstrate whether *Photorhabdus* has different mechanisms of pathogenicity dependent on the insect host used.

It was suggested in this study that flagella mediated motility may contribute towards nematode colonisation by facilitating biofilm formation. This was based on the data that *Photorhabdus* forms intimate associations with the nematode gut cells as well as other *Photorhabdus* cells (ffrench-Constant *et al.*, 2003). The mutant BMM316 was deficient in forming a biofilm *in vitro*; therefore a fully motile biofilm-deficient mutant should be generated and tested *in vivo* for colonisation of *Heterorhabditis*. This experiment would determine whether the colonisation defect seen in BMM316 is due to the absence of flagella or the slightly slower biofilm formation.

Interestingly, although it appears that motility is correlated with phenotypic variation (Givaudan *et al.*, 1995) recent work during the course of this study has shown it may be regulated separately. A HexA secondary variant mutant was shown to up regulate many primary characteristics, however motility was unaffected (Joyce and Clarke, 2003). Anaerobic conditions were shown to restore motility to a secondary variant independent of almost all other characteristics (Hodgson *et al.*, 2003). A primary variant *Xenorhabdus rpoS* mutant was shown to up regulate motility independently of other primary characteristics (Vivas and Goodrich-Blair, 2002). Lastly a two-component pathway AstRS has been shown to down regulate motility whilst conversely up regulating other primary characteristics (Derzelle *et al.*, 2004b). Therefore, rather than divergent regulation of one master gene controlling many primary characteristics, it may be that many regulatory mechanisms converge together to control the primary to secondary variation. Discovering the regulatory pathways involved in controlling motility in *Photorhabdus* will further contribute to understanding the role of motility in the *Photorhabdus* life cycle.

During the course of this study into the effect of motility on the host interactions of *Photorhabdus* a mutant, BMM305, was characterised and shown to be defective in the colonisation of both hosts. The mutant BMM305 lacked an O antigen and during this study several hypothesises have been put forward as to the putative role of O antigen in both host interactions.

One idea suggested that the avirulent nature of BMM305 towards *G. mellonella* was due to a lack of growth and that O antigen conferred the ability to grow *in vivo*; in order to test this hypothesis parental LPS could be co-injected with BMM305. This would differentiate between the idea that LPS release causes damage and death and that the presence of LPS confers resistance to insect host mediated stresses. A lack of O antigen also led to an inability to successfully colonise the nematode host. It is unknown how the O antigen confers symbiotic potential. It may provide resistance to stresses inside the nematode, confer a recognisable cell surface feature for symbiont detection or act as a signal for symbiosis. LPS prepared from *Photorhabdus temperata* K122 demonstrated a different banding pattern on SDS PAGE suggesting differences between the O antigen of K122 and TT01 (Fig. 5.4 D). This structural variation could present a basis for specificity amongst *Photorhabdus/Heterorhabditis* symbiotic partnerships. It would be interesting to knockout LPS genes from one *Photorhabdus* species and replace them with cloned genes from a second and examine whether specificity for the nematode partner is conferred. Taken further this could lead to the creation of new symbiotic partnerships and the ability to match nematode host finding behaviour with host pathogenicity to create a specific biocontrol agent.

The complementation of the colonisation defect of *pbgE1* was not performed during this study due to the fact that upon incubation on lipid agar plates TT01 and BMM305 lost non-essential plasmids. As *pbgE1* is not essential for nematode growth and development it is not retained for this purpose and for the 3 weeks incubation time, needed to allow nematode growth and development, antibiotic selection could not be maintained. Therefore it would be necessary to reintroduce *pbgE1* back onto the chromosome in order to conclusively prove it restores colonisation to BMM305.

The role of the cell surface structures, flagella and LPS, in the life cycle of *Photorhabdus* has been highlighted in this study. However the *Photorhabdus* genome also contains genes that suggest it manufactures various pili structures, and a recent study has examined the regulation of fimbriae, although not the biological role (Duchaud *et al.*, 2003; Meslet-Cladiere *et al.*, 2004). Furthermore *Photorhabdus* produces a thick capsule (Brehélin *et al.*, 1993) and all these cell-surface associated structures will be in close contact with both insect and nematode host cells suggesting that they may also have biological functions in host interactions and represent further targets for research into this field.

The genes necessary for the host interactions of *Photorhabdus* can be divided into three groups; those required for 1) pathogenicity, 2) supporting the development of the nematode partner and 3) colonisation and survival inside the nematode intestine (Fig. 6.0). However there is some overlap in these areas such as general housekeeping genes that would be required for metabolism. Progress has been made into understanding the pathogenic nature of *Photorhabdus* with several recent studies examining the toxin complexes and discovering novel genes for exploitation in insect control (*mcf*) (Liu *et al.*, 2003; Daborn *et al.*, 2002). Further interesting research by Joyce and Clarke (2003) has helped bring forward understanding of the mechanisms of phenotypic variation, and consequently symbiosis, by examining gene regulators (*hexA*). This research and other work in this field is important for the commercial use of nematodes as the appearance of secondary, non-symbiotic, variants greatly affects production yields (Ehlers, 2001).

This study set out to further the knowledge in the fields of both *Photorhabdus* pathogenic and symbiotic host associations by identifying important genes involved in these interactions. To this end it was successful, identifying for the first time in *Photorhabdus* characteristics important for colonisation of *Heterorhabditis* (motility and O antigen). Furthermore it identified *pbgE1* (and therefore O antigen) as important for colonisation of the insect host, *G. mellonella*, the first gene identified so far in *Photorhabdus* as essential for interactions with both hosts.

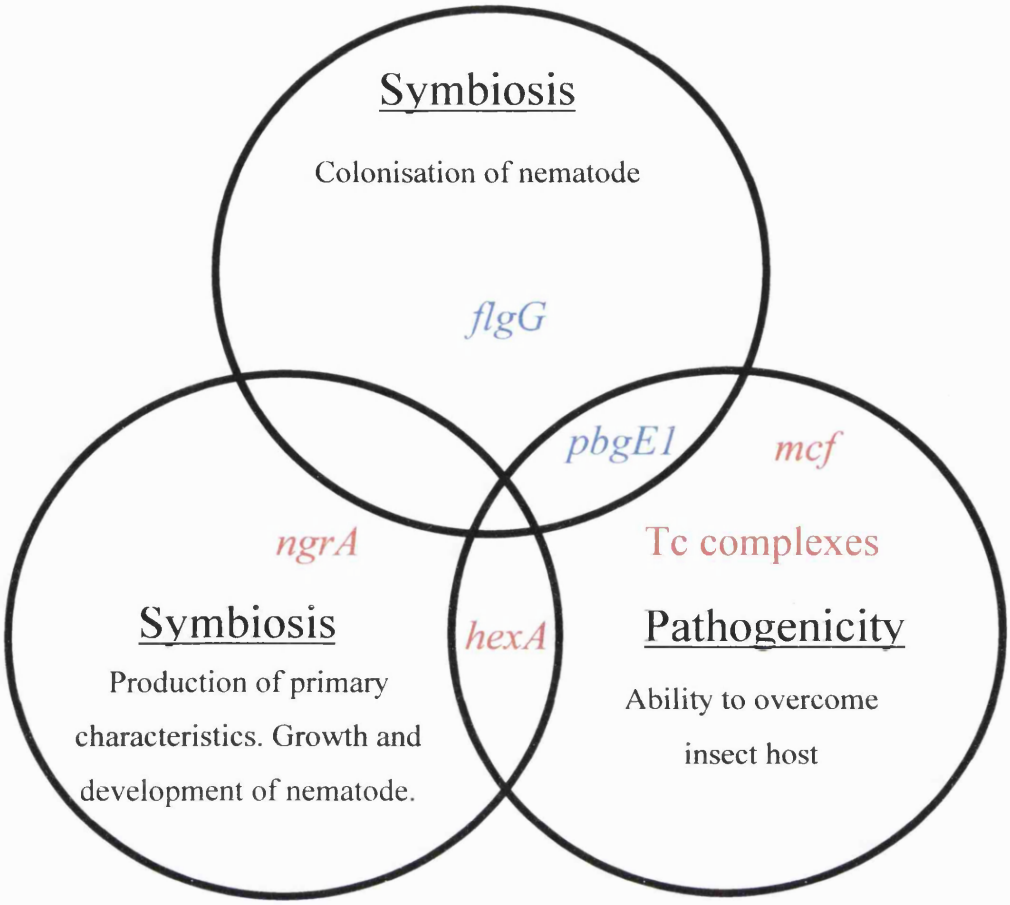

Figure 6.0. Diagram showing some of the genes and characteristics so far identified as being important for pathogenicity or symbiosis of *Photorhabdus*. Words in blue represent factors identified in this study, words in red represent factors identified in other studies.

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